



Summer 8-14-2009

# Rs Rearrangement: Observations and Implications From a Novel Assay of B Cell Tolerance

Anil Kumar Panigrahi

University of Pennsylvania, [anilpani@mail.med.upenn.edu](mailto:anilpani@mail.med.upenn.edu)

Follow this and additional works at: <http://repository.upenn.edu/edissertations>

 Part of the [Medical Cell Biology Commons](#), [Medical Immunology Commons](#), and the [Medical Molecular Biology Commons](#)

---

## Recommended Citation

Panigrahi, Anil Kumar, "Rs Rearrangement: Observations and Implications From a Novel Assay of B Cell Tolerance" (2009). *Publicly Accessible Penn Dissertations*. 2.

<http://repository.upenn.edu/edissertations/2>

This paper is posted at ScholarlyCommons. <http://repository.upenn.edu/edissertations/2>

For more information, please contact [libraryrepository@pobox.upenn.edu](mailto:libraryrepository@pobox.upenn.edu).

---

# Rs Rearrangement: Observations and Implications From a Novel Assay of B Cell Tolerance

## **Abstract**

Antibody diversity is generated through the random recombination of immunoglobulin gene segments. As a consequence of this stochastic process, antibodies recognizing self-antigens can also be produced. Continued antibody gene rearrangement, termed receptor editing, is an important mechanism of central B cell tolerance that serves to alter the antibody specificity of developing B cells. Although it has been demonstrated to be critical for normal B cell development, the role of receptor editing in the context of autoimmune disease and the forces that stimulate receptor editing are not fully understood. To address these issues, a novel quantitative assay based upon Recombining Sequence (RS) recombination was developed to measure receptor editing rearrangements in B cell populations. RS rearrangement is a recombination of a non-coding gene segment in the immunoglobulin  $\kappa$  light chain locus. Unlike other markers of receptor editing, the RS rearrangement assay does not rely upon expressed components of the B cell receptor and therefore is not restricted to specific autoantigens.

As receptor editing may be defective in some autoimmune individuals, the RS assay was applied to autoimmune mouse models of systemic lupus erythematosus (SLE) and type 1 diabetes (T1D), which demonstrated decreased receptor editing levels relative to wild-type mice. Low RS rearrangement levels were also observed in human subjects with SLE or T1D, suggesting that defects in receptor editing may contribute to disease susceptibility. Additionally, RS rearrangement was used to assess the role of self-antigen mediated B cell signaling in the stimulation of receptor editing. B cells expressing an anti-DNA heavy chain were found to undergo more extensive receptor editing and functional precursors of editing were found to be biased towards autoreactivity, indicating that self-reactivity may promote receptor editing. To begin to examine whether positive signaling is required for receptor editing, the roles of the inhibitory kinase Lyn and a disease associated variant of the lymphoid phosphatase PTPN22 were analyzed. The results indicate that aberrant activity of these signaling components alone does not influence receptor editing. Collectively the findings described in this dissertation demonstrate that RS rearrangement is an effective means of estimating receptor editing that may be used to monitor B cell central tolerance and further evaluate the regulation of editing rearrangement.

## **Degree Type**

Dissertation

## **Degree Name**

Doctor of Philosophy (PhD)

## **Graduate Group**

Cell & Molecular Biology

## **First Advisor**

Eline T. Luning Prak

---

**Keywords**

receptor editing, RS recombination, lupus, type 1 diabetes, autoimmunity

**Subject Categories**

Medical Cell Biology | Medical Immunology | Medical Molecular Biology

**RS REARRANGEMENT: OBSERVATIONS AND IMPLICATIONS FROM A  
NOVEL ASSAY OF B CELL TOLERANCE**

**Anil K. Panigrahi**

**A DISSERTATION  
in  
Cell and Molecular Biology**

**Presented to the Faculties of the University of Pennsylvania**

**In Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**2009**

  
**Dissertation Supervisor**

  
**Graduate Group Chairperson**



*For my father,  
who cultivated my interest in science.*

## ACKNOWLEDGEMENTS

First and foremost I would like to thank my mentor Nina Luning Prak for her support and guidance. Her enthusiasm for science and dedication to teaching are unparalleled and have made me a better scientist.

I would also like to thank my thesis committee: Bryan Wolf for generously serving as the chair, David Allman, Yair Argon, and Youhai Chen for their time and constructive criticism. I also thank John Monroe for helpful discussions and advice.

I thank Ali Naji, Robert Eisenberg, Michael Rickels, Andrew Gross, Almut Meyer-Bahlburg, Shirly Herman, Archana Brahmandam, David Rawlings, and Jane Buckner for collaboration in this work. I also thank Phil Cohen, Hooman Noorchahsm, and Jan Erikson for reagents and useful suggestions.

Additionally, I would like to thank Maggie Krall, Mary Tiedeman, Maureen Osciak, and Skip Brass in the MD/PhD Program office for their support.

I am also indebted to past and present members of the Luning Prak lab, Noah Goodman, Lenka Yunk, Sara Smith, Wenzhao Meng, Yangzhu Du, Jennifer Sutter, Andrew Fesnak, Peffin Lee, Deep Charan, Janet Salit, Yang Liu, Laura Heckman, Shane Horman and Debora Sekiguchi for their camaraderie and technical assistance.

Finally, I would like to thank my family, Mom, Dad, Eva, and Sridhar Uncle for their unconditional love and support. I also thank the Lefterov family for their support and encouragement. To Martina thank you for your love, patience and guidance throughout this process. Without you this would not be possible.

# **ABSTRACT**

## **RS REARRANGEMENT: OBSERVATIONS AND IMPLICATIONS FROM A NOVEL ASSAY OF B CELL TOLERANCE**

Anil K. Panigrahi

Eline T. Luning Prak

Antibody diversity is generated through the random recombination of immunoglobulin gene segments. As a consequence of this stochastic process, antibodies recognizing self-antigens can also be produced. Continued antibody gene rearrangement, termed receptor editing, is an important mechanism of central B cell tolerance that serves to alter the antibody specificity of developing B cells. Although it has been demonstrated to be critical for normal B cell development, the role of receptor editing in the context of autoimmune disease and the forces that stimulate receptor editing are not fully understood. To address these issues, a novel quantitative assay based upon Recombining Sequence (RS) recombination was developed to measure receptor editing rearrangements in B cell populations. RS rearrangement is a recombination of a non-coding gene segment in the immunoglobulin  $\kappa$  light chain locus. Unlike other markers of receptor editing, the RS rearrangement assay does not rely upon expressed components of the B cell receptor and therefore is not restricted to specific autoantigens.

As receptor editing may be defective in some autoimmune individuals, the RS assay was applied to autoimmune mouse models of systemic lupus erythematosus (SLE) and type 1 diabetes (T1D), which demonstrated decreased receptor editing levels

relative to wild-type mice. Low RS rearrangement levels were also observed in human subjects with SLE or T1D, suggesting that defects in receptor editing may contribute to disease susceptibility. Additionally, RS rearrangement was used to assess the role of self-antigen mediated B cell signaling in the stimulation of receptor editing. B cells expressing an anti-DNA heavy chain were found to undergo more extensive receptor editing and functional precursors of editing were found to be biased towards autoreactivity, indicating that self-reactivity may promote receptor editing. To begin to examine whether positive signaling is required for receptor editing, the roles of the inhibitory kinase Lyn and a disease associated variant of the lymphoid phosphatase PTPN22 were analyzed. The results indicate that aberrant activity of these signaling components alone does not influence receptor editing. Collectively the findings described in this dissertation demonstrate that RS rearrangement is an effective means of estimating receptor editing that may be used to monitor B cell central tolerance and further evaluate the regulation of editing rearrangement.

# TABLE OF CONTENTS

Dedication	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	vi
List of Figures and Tables	vii
List of Abbreviations	viii
<b>CHAPTER 1: GENERAL INTRODUCTION</b>	<b>1</b>
<i>B Cell Development</i>	2
<i>Regulation of V(D)J Recombination</i>	5
<i>Pre-B Cell Receptor Mediated Regulation of B Cell Development</i>	11
<i>Mechanisms of Immunological Tolerance</i>	17
<b>CHAPTER 2: RS REARRANGEMENT FREQUENCY AS A MARKER OF RECEPTOR EDITING IN LUPUS AND TYPE 1 DIABETES</b>	<b>31</b>
INTRODUCTION	32
RESULTS	34
<i>A novel assay for estimating levels of receptor editing</i>	34
<i>RS rearrangement levels vary between developmental and functional B cell subsets in mice</i>	35
<i>Mouse strains prone to autoimmunity display lower levels of RS rearrangement</i>	36
<i>RS rearrangement levels are lower in human SLE and T1D</i>	37
DISCUSSION	39
<b>CHAPTER 3: INVESTIGATION OF THE FORCES THAT REGULATE RECEPTOR EDITING</b>	<b>59</b>
INTRODUCTION	60
RESULTS	61
<i>RS rearrangement levels differ between autoreactive and non-autoreactive B cells</i>	61
<i>Recovery of editing precursors from splenic B cells</i>	62
<i>Analysis of editing precursors</i>	65
<i>Identifying editing precursors among reciprocal products</i>	66
<i>Lyn-mediated inhibition of BCR signaling does not influence receptor editing</i>	68
<i>Autoimmune associated polymorphism in PTPN22 does not affect receptor editing</i>	68
<i>NOD Idd loci 3 and 5 do not contribute to decreased RS rearrangement levels</i>	70
DISCUSSION	71
<b>CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS</b>	<b>93</b>
SUMMARY	94
FUTURE DIRECTIONS	96
<i>Development of RS Rearrangement as a Clinical Assay</i>	96
<i>RS Rearrangement Assay Optimization</i>	98
<i>Control of RS Recombination</i>	99
<i>The Significance of Altered RS Recombination</i>	103
<b>CHAPTER 5: MATERIALS AND METHODS</b>	<b>109</b>
Mice	110
Flow Cytometry	110
Quantitative PCR	111
V <sub>K</sub> -RS and iRS-KDE Standard Curve	112
Human Subjects	113
RS Precursor Isolation	113
Insulin Staining of Fresh Splenocytes for Flow Cytometric Analysis	114
Statistical Analysis	115
<b>REFERENCES</b>	<b>116</b>

## LIST OF FIGURES AND TABLES

Figure 1-1: B cell development is an ordered process involving progressive stages of maturation defined by changes in protein expression and gene transcription.....	26
Figure 1-2: Pre-B cell receptor signaling regulates pre-B cell proliferation and differentiation. ...	27
Figure 1-3: Schematic of the germline murine Ig $\kappa$ locus. ....	29
Figure 1-4: Receptor editing at the Ig $\kappa$ locus.....	30
Figure 2-1: RS rearrangement is a marker of extensive $\kappa$ light chain rearrangement.....	44
Figure 2-2: Flow cytometric analysis of murine and human lymphocytes. ....	46
Figure 2-3: Real-time PCR to monitor iRS-KDE rearrangements in B cell populations. ....	48
Figure 2-4: RS rearrangement levels amongst $\kappa^+$ B cells are independent of Ig $\kappa$ /Ig $\lambda$ ratios. ....	49
Figure 2-5: RS rearrangement levels are highest in late pre-B cells.....	51
Figure 2-6: Autoimmune prone mouse strains display reduced levels of RS rearrangement.....	52
Figure 2-7: RS rearrangement levels in MRL/MpJ and NOR strains.....	53
Figure 2-8: RS rearrangement levels do not correlate with patient group demographics.....	54
Figure 2-9: RS rearrangement levels are lower amongst SLE and T1D patients. ....	56
Table 2-1: Demographic comparison among subject groups. ....	58
Figure 3-1: RS rearrangements are increased in B cells expressing an autoreactive heavy chain in self tolerant mice.....	79
Figure 3-2: Multiple editing rearrangement pathways are possible at the murine Ig $\kappa$ locus. ....	80
Figure 3-3: iRS-RS junctions demonstrate a high degree of diversity. ....	82
Figure 3-4: A high frequency of editing precursors on RS deleted kappa alleles are in-frame.....	83
Figure 3-5: Absence of Lyn does not impair receptor editing and clonal deletion of immature B cells. ....	87
Figure 3-6: The PTPN22 1858T variant does not affect receptor editing in healthy individuals. ...	88
Figure 3-7: AEC mice display RS rearrangement levels equivalent to wild-type mice. ....	90
Table 3-1: Three models of receptor editing predict different frequencies of in-frame editing precursors.....	91
Table 3-2: Nucleotide sequences at the junctions of inverted V $\kappa$ -J $\kappa$ 1 rearrangements reveal several IF editing precursors.....	92
Figure 4-1: Bcl-x $_L$ expression increases V $\kappa$ -RS rearrangement levels in immature bone marrow B cells. ....	106
Figure 4-2: Auto-antigen staining of C57Bl/6 and 56R $^{+/-}$ splenic B cells.....	107

## LIST OF ABBREVIATIONS

3'E <sub>κ</sub>	3' kappa enhancer – located downstream of C <sub>κ</sub>
AEC	autoimmune exocrinopathy
BCR	B cell receptor
BLyS	B lymphocyte stimulator
CLP	common lymphoid progenitor
Fr.	Bone marrow fraction as defined by (Hardy et al. 1991)
Fo	Follicular B cell
Foxo	FOX class O transcription factor
HEL	hen egg lysozyme
Idd	insulin dependent diabetes (genetic locus)
iE <sub>κ</sub>	intronic kappa enhancer – located in the J <sub>κ</sub> –C <sub>κ</sub> intron
IF	in (reading) frame
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
Ig <sub>κ</sub>	kappa light chain
IgL	immunoglobulin light chain
Ig <sub>λ</sub>	lambda light chain
IL-7	interleukin 7
IRF-4	interferon regulatory factor 4
iRS	intron RS
KDE	kappa deleting element
MZ	marginal zone B cell
NOD	non-obese diabetic
NOR	non-obese resistant
OF	out of (reading) frame
PDK1	3-phosphoinositide dependent kinase 1
PI3K	phosphoinositide-3 kinase
PLC <sub>γ</sub> 2	phospholipase C <sub>γ</sub> 2
pre-BCR	pre-B cell receptor
PTPN22	protein tyrosine phosphatase non-receptor 22
RA	rheumatoid arthritis
RAG	recombinase activating gene
RP	reciprocal product
RS	recombining sequence
RSS	recombination signal sequence
sd-tg	site-directed transgene
SLC	surrogate light chain
SLE	systemic lupus erythematosus
SLP65	SH2-domain containing leukocyte protein of 65kDa
SNP	single nucleotide polymorphism
Syk	spleen tyrosine kinase
T1D	type 1 diabetes
TCR	T cell receptor
tg	transgene

## **CHAPTER 1: General Introduction**



In order to recognize the vast array of antigens encountered by the immune system, B cells undergo a stochastic process of gene rearrangement to generate a diverse repertoire of unique receptors. Immunoglobulin gene rearrangement, also termed V(D)J recombination, is highly regulated to ensure the efficient generation of B cells that carry multiple copies a single B cell receptor. Due to the random nature of this process, an inevitable consequence is the production of self-reactive B cells. In response to the development of autoreactive B cells, tolerance checkpoints exist at several stages of B cell development to remove or alter these cells and thereby prevent the development of autoimmunity. In this chapter I will begin with a brief review of B cell development and will follow with a discussion of the regulation of immunoglobulin gene rearrangement, highlighting key regulatory mechanisms that act both genetically and at the protein level. Finally, the chapter will conclude with a discussion of the prevalence of autoreactive B cells and the immunological tolerance mechanisms that serve to silence them.

## **B Cell Development**

B cells develop from hematopoietic stem cells through a series of differentiative steps. During fetal and neonatal life murine B cell development begins in the liver with the generation of B1a B cells (Hayakawa et al. 1985). These B cells, which are IgM<sup>hi</sup> IgD<sup>lo</sup> CD11b<sup>+</sup> CD5<sup>+</sup> (Dorshkind and Montecino-Rodriguez 2007), normally populate the pleural and peritoneal cavities and express a limited repertoire of immunoglobulin genes (Yancopoulos et al. 1988). The restricted use of D<sub>H</sub>-proximal V<sub>H</sub> genes results in a decreased ability of B1a cells to respond to diverse antigens. However, B1a B cells have been shown to secrete “natural antibodies” which are mainly of the IgM isotype and are polyreactive, recognizing common bacterial and viral antigens as well as self-antigens (Hayakawa et al. 1984; Hayakawa et al. 2003). Consequently, B1a B cells have

been proposed to function as links between innate and adaptive immune responses by providing a quickly activated non-specific humoral response.

In mature mice, B cell development takes place in the bone marrow where hematopoietic stem cells (HSCs) gradually lose their ability to form other cell types and become committed to a B cell fate (**Fig. 1–1**). Multipotent progenitors (MPPs) are the most HSC-proximal cell type to show evidence of B lineage specification with a subset of MPPs expressing the RAG1 and RAG2 genes (Igarashi et al. 2002; Rumfelt et al. 2006). MPPs then give rise to common lymphoid progenitors (CLPs) which act as a key source of B-lineage committed cells. CLPs express high levels of B cell specific genes and are the earliest cell type to express the IL-7 receptor, marking this as a critical stage in B cell development (Miller et al. 2002). CLPs generate pre-pro B cells (Hardy fraction A) that are CD45R/B220<sup>+</sup> (hereafter B220) but lack CD19 expression. The vast majority of these cells (~75%) carry D<sub>H</sub>J<sub>H</sub> rearrangements on both alleles and express high levels of B cell transcription factors Pax5 and EBF (Li et al. 1996), indicating their high degree of B cell commitment. Expression of Pax5 and EBF activates the B cell transcriptional program in earnest leading to development of pro-B cells (Hardy fraction B) that are fully committed to the B lineage (Nutt et al. 1999; Pongubala et al. 2008). Fraction (Fr.) B pro-B cells lead to early pre-B cells (Hardy Fr. C) which complete V<sub>H</sub> to D<sub>H</sub>J<sub>H</sub> rearrangement and assemble the pre-B cell receptor (pre-BCR) at the cell surface (Hardy et al. 1991; Nishimoto et al. 1991). Signaling via the pre-BCR results in several rounds of cell division as well as pre-BCR downregulation via a negative-feedback loop (Mundt et al. 2001; Parker et al. 2005). Following proliferation, late pre-B cells (Hardy Fr. D), which no longer express surface immunoglobulin, commence light chain gene rearrangement. Productive light chain rearrangement leads to light chain expression and assembly of the B cell receptor (BCR) on the surface of immature B cells (Hardy Fr. E). These newly formed immature B cells can continue development in the bone marrow

(Lindsley et al. 2007) or transit to the spleen where they continue development as transitional B cells (Loder et al. 1999).

Although there is increasing evidence for the maturation of transitional B cells in the bone marrow (Cariappa et al. 2007), B cell maturation has been more rigorously characterized in the periphery where transitional B cells have been defined in multiple developmental schemes (Loder et al. 1999; Allman et al. 2001). In this dissertation I will use the stages described by Allman *et al.*, which separate transitional B cells into three distinct subsets, T1, T2, and T3. B cells in all three transitional subsets demonstrate characteristics of immaturity including failure to proliferate in response to BCR signaling, rapid turnover rate, and expression of the cell surface marker CD93/AA4 (Rolink et al. 1998; Allman et al. 2001). The T1 subset represents the least mature population of transitional cells as demonstrated by the absence of CD23 and IgD expression. Adoptive transfer and BrdU labeling studies indicate that T1 cells give rise to T2 cells which begin to express both CD23 and IgD (Loder et al. 1999; Allman et al. 2001). The T3 subset has been proposed as an intermediate stage between T2 transitional cells and mature B cells. Similar to mature B cells, T3 B cells express lower levels of surface IgM, however, they still retain CD93/AA4 expression marking their immaturity. Studies by Cambier and colleagues have suggested that the T3 subset is enriched for autoreactive B cells that have entered an anergic state (Merrell et al. 2006). These cells were found to be unresponsive to BCR stimulation as measured by intracellular  $\text{Ca}^{2+}$  levels and protein tyrosine phosphorylation (Cooke et al. 1994; Benschop et al. 2001).

After progressing through transitional stages, mature B cells ultimately enter one of two mature splenic B cell populations. Most developing cells enter the follicular (Fo) pool of mature cells which are so termed due to their location in follicles within peripheral lymphoid organs. These fully mature cells exist in a resting state for 15–20

weeks (Rolink et al. 1998) and are found primarily in the spleen, but also circulate in the blood and lymph. Rajewsky and colleagues demonstrated that survival of these cells is dependent upon BCR signaling (Lam et al. 1997; Kraus et al. 2004), however, the nature of this signal as tonic, basal signaling or antigen-dependent positive selection remains unresolved.

Marginal zone (MZ) B cells comprise the second group of mature B cells that, unlike Fo B cells, do not circulate and instead reside primarily in regions surrounding marginal sinuses in the spleen. These sinuses act as a major site of blood filtration and antigen presentation, thus making MZ B cells uniquely positioned to act in a primary response to blood borne pathogens. Indeed, MZ B cells, much like B1 B cells, express a restricted repertoire of BCRs that are biased toward recognition of bacterial cell wall components, such as phosphorylcholine (Martin and Kearney 2000), and rapidly proliferate and differentiate into IgM-secreting plasmablasts in response to lipopolysaccharide stimulation (Oliver et al. 1999; Martin et al. 2001). The recruitment of newly formed B cells into the marginal zone population is still not well characterized. Based on BCR repertoire bias as well as the finding of preferential recruitment of autoreactive B cells to the marginal zone (Martin and Kearney 2000; Li et al. 2002), it has been proposed that weakly self-reactive or poly-reactive B cells are selected into the MZ through interactions with self-antigen (Hayakawa et al. 1999; Dammers et al. 2000). Whether this is the primary pathway of MZ B cell development is still unclear.

### **Regulation of V(D)J Recombination**

In order to generate competent B cells, B cell development is regulated at several checkpoints. An important area of B cell regulation involves control of V(D)J recombination which is responsible for generating functional BCRs. Both the BCR and the T cell receptor (TCR) are produced via rearrangement of gene segments in the  $\mu$ ,  $\kappa$ ,

$\lambda$ ; and  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  loci, respectively. Recombination of these gene segments is catalyzed by a protein complex that includes the lymphocyte-specific gene products of recombinase activating genes 1 and 2 (RAG1 and RAG2). The RAG proteins combine to form a heteromultimeric complex that creates double strand DNA breaks at precise locations flanking immunoglobulin gene segments marked by conserved recombination signal sequences (RSSs). These breaks are then resolved by additional components of the RAG complex which are common to the non-homologous end-joining (NHEJ) repair pathway (Taccioli et al. 1993; Taccioli et al. 1994; Blunt et al. 1995).

As the RAG genes are the primary component of the recombinase machinery restricted to lymphocytes, they are highly regulated to ensure that V(D)J recombination is limited to these cells. Furthermore, RAG expression varies throughout development, providing an additional level of control over gene rearrangement. As discussed above, the RAG proteins are first expressed in a subset of MPPs and levels continue to rise as the cells develop into pro-B cells (Hardy Fr. B) where immunoglobulin heavy chain (IgH) gene recombination begins. Successful V(D)J rearrangement yields an IgH variable region with an intact reading frame and assembly of a complete IgH that is capable of pairing with the surrogate light chain (SLC) and forming the pre-B cell receptor (pre-BCR). Surface expression of the pre-BCR results in down-regulation of RAG transcription and cessation of further rearrangement (Grawunder et al. 1995). Pre-B cells then undergo a proliferative burst after which RAG is re-expressed and immunoglobulin light chain rearrangement (IgL) begins. Generation of a functional IgL and assembly of the BCR on the cell surface ultimately leads to termination of RAG expression and gene rearrangement in immature B cells. Thus RAG expression is restricted to developmental stages during which V(D)J recombination occurs. This regulation occurs via transcriptional and post-transcriptional mechanisms.

The RAG1 and RAG2 genes are each expressed from single exons that are convergently transcribed. Promoter elements for both genes have been characterized in mouse and man (Fuller and Storb 1997; Zarrin et al. 1997). Although the RAG1 promoter appears to be active in non-lymphoid cells, RAG2 promoter activity is lymphoid restricted (Lauring and Schlissel 1999). Several transcription factors have been proposed to bind RAG promoters and enhance their activity, including c-myb and Pax5 (Kishi et al. 2002), lymphoid enhancer binding factor-1 (LEF-1; (Jin et al. 2002)), and NF- $\kappa$ B (Brown, Miranda et al. 1997). Furthermore, B and T cells display differential RAG2 promoter binding, with Pax5 and GATA3 binding in B and T cells, respectively.

In addition to the promoter regions, distal regulatory elements have also been found to modify RAG expression. Studies using BAC reporter constructs and embryonic stem cell transfection assays demonstrated that RAG expression in developing B and T cells was independent of sequences 5' of RAG1, but required sequences within 10kb 5' of the RAG2 promoter (Monroe et al. 1999; Yu et al. 1999). Additional reports have characterized several enhancers that have been shown to activate RAG expression in immature lymphoid cells. The RAG locus enhancer D3, located ~8kb 5' of the RAG2 gene, contains binding sites for several lymphoid transcription factors, including c-myb, c/EBP, GATA3, and NF- $\kappa$ B, and was shown to activate expression in pre-T and pre-B cells but have minimal activity in mature lymphocytes and non-lymphoid cells (Wei et al. 2002). Another enhancer element, ERAG, located 22kb 5' of the RAG2 promoter, has been shown to regulate RAG expression in B cells specifically. ERAG activated RAG expression in pro-B cell lines and targeted deletion resulted in a partial block in B cell development at the pro-B cell stage (Hsu et al. 2003). Interestingly, T cell development was unaffected suggesting that ERAG function is restricted to developing B cells.

Although temporal and lineage regulation of RAG expression restricts V(D)J recombination to lymphocytes at specific stages in development, these mechanisms

cannot account for the locus preference observed in these various cell types. For instance, in B cells recombination is limited to immunoglobulin genes, while TCR genes rearrange in T cells. Furthermore, these rearrangements are ordered such that IgH and TCR $\beta$  genes rearrange before IgL and TCR $\alpha$  genes in B and T cells, respectively. It is now becoming evident that changes in chromatin structure contribute to this additional level of regulation. The initial clues implicating chromatin modification come from early studies of Ig gene rearrangement that showed increased transcription levels of unrearranged gene segments in both the IgH (Yancopoulos and Alt 1985) and IgL (Schlissel and Baltimore 1989) loci that correlated with their rearrangement. These findings supported a model where chromatin modifications preceded gene rearrangement making the loci accessible, thus promoting transcription and rearrangement.

DNA methylation has been proposed as a mechanism that may contribute to RAG substrate accessibility. Studies of *in vitro* substrates have found that methylation of CpG dinucleotides inhibits RAG mediated cleavage of RSSs (Engler et al. 1991; Hsieh and Lieber 1992). These findings were also confirmed *in vivo* where TCR $\beta$  recombination was shown to occur preferentially on hypomethylated alleles (Whitehurst et al. 2000). Changes in methylation that coincide with developmental patterns of V(D)J rearrangement have also been described. Experiments by Bergman and colleagues demonstrated monoallelic demethylation of the immunoglobulin kappa (Ig $\kappa$ ) locus in pre-B cells prior to rearrangement (Mostoslavsky et al. 1998). Together these data support a role for DNA methylation in limiting gene rearrangement, however, more recent studies have questioned the validity of this model. Induced inactivation in thymocytes of DNA methyltransferase 1 (Dnmt1), an enzyme responsible for adding methyl groups to cytosine residues, did not adversely affect the temporal pattern of TCR

gene rearrangement and suggests that additional methyltransferases or other regulatory mechanisms are involved.

Histone modifications are a second type of chromatin mark that has been proposed to regulate RAG accessibility. An indication of the importance of nucleosome structure in regulating V(D)J recombination came from *in vitro* cleavage studies of RAG substrates purified from different types of lymphocytes. Schlissel and colleagues demonstrated that recombinant RAG was capable of cleaving RSSs from purified genomic DNA in a lineage-independent manner, however, DNA from nuclei of pro-B and pro-T cell lines only underwent rearrangement at lineage appropriate loci (Stanhope-Baker et al. 1996). Further studies determined that assembly of RAG substrates onto nucleosomes inhibited RSS cleavage indicating that changes in the chromatin landscape likely influence RAG accessibility (Kwon et al. 1998; Golding et al. 1999). Consistent with this idea, histone acetylation and remodeling were found to enhance RAG mediated cleavage (Kwon et al. 2000). Additionally, acetylation of histones was shown to be dynamic and correlated with developmental changes in RSS accessibility both in T and B cells (McMurry and Krangel 2000; Chowdhury and Sen 2001). Interestingly, changes in histone acetylation have been shown to be dependent upon enhancer activity (Mathieu et al. 2000), providing a link between germline transcription and RAG accessibility. Other histone modifications are also likely involved in controlling V(D)J recombination, as conditional mutation of the histone methyltransferase Ezh2 inhibits  $V_H$ -to- $D_HJ_H$  recombination of 5'  $V_H$  gene segments in pro-B cells and impairs their progression to the pre-B cell stage (Su et al. 2003).

Despite the substantial evidence for the role of histone modifications in the regulation of V(D)J recombination, subsequent studies suggest that these changes alone are insufficient to regulate accessibility. For instance, the decreased histone methylation caused by Ezh2 mutation had no effect on germline transcription and



histone acetylation of the IgH locus in pro-B cells (Su et al. 2003). Moreover, experiments monitoring TCR $\beta$  loci stably transfected in lymphocytes found that germline transcription and histone acetylation were insufficient to promote recombination (Sikes et al. 2002). Similar findings were observed in B cells where Pax5<sup>-/-</sup> pro-B cells failed to rearrange 5' V<sub>H</sub> gene segments despite their accessibility as measured by germline transcription and histone acetylation (Hesslein et al. 2003). Together these data indicate that although they are highly correlated with accessible chromatin, histone modifications alone are insufficient to regulate V(D)J rearrangement and highlight the multifaceted nature of recombinase regulation.

Recent studies have introduced nuclear sublocalization as an additional mechanism for regulation of gene expression. Consequently, this process has been studied to determine if it may contribute to regulation of V(D)J rearrangement. Nuclear studies in B cells revealed that transcriptionally repressed genes co-localized with the lymphoid-specific transcription factor Ikaros in centromeric heterochromatin, suggesting a role in the lineage restriction of lymphoid gene expression (Brown, Guest et al. 1997). Additional experiments revealed that in mature B cells unexpressed IgH and IgL alleles were associated with heterochromatin, while expressed alleles were not (Skok et al. 2001). More direct evidence for the role of nuclear compartmentalization in the process of V(D)J recombination came from subsequent work in developing B cells. Kosak *et al.* found that in embryonic stem cells, MPPs, and pro-T cells the IgH and Ig $\kappa$  loci are positioned in the nuclear periphery, however, in pro-B cells the loci are centrally located (Kosak et al. 2002). Additionally, the authors observed that once positioned centrally in the nucleus, the IgH locus underwent compaction that was restricted to pro-B cells suggesting that this process may facilitate recombination of distal V<sub>H</sub> genes.

## **Pre-B Cell Receptor Mediated Regulation of B Cell Development**

Expression of the pre-BCR represents another critical checkpoint in B cell development. As discussed above, pre-BCR signaling serves to stop IgH recombination and stimulate pre-B cell proliferation. Paradoxically, pre-BCR signaling is also involved in promoting IgL gene rearrangement (Lu et al. 2003; Herzog et al. 2008; Johnson et al. 2008; Xu and Feeney 2009). Due to the temporal proximity of these events, pre-BCR expression and signaling are strictly controlled in order to prevent genomic instability and cellular transformation. The pre-BCR is expressed at the pre-B cell stage and is comprised of two IgH chains that each pair with a surrogate light chain (SLC). The SLC itself is a complex of two proteins,  $\lambda 5$  and VpreB. The paired IgH and SLC represent the extracellular components of the pre-BCR that associate with  $Ig\alpha$  and  $Ig\beta$ , which serve to propagate signals within the cell. The requirement of pre-BCR signaling for B cell development was conclusively demonstrated in studies by the Rajewsky lab that generated mice with a targeted deletion of the transmembrane region of  $Ig\mu$  (IgH of IgM), causing disruption of its surface expression (Kitamura et al. 1991). The loss of surface  $Ig\mu$  resulted in a block in B cell development at the pro-B stage and the absence of mature B cells. Additional experiments demonstrated similar B cell developmental defects due to the loss of  $Ig\alpha$  and  $Ig\beta$  (Gong and Nussenzweig 1996; Pelanda et al. 2002) as well as SLC components  $\lambda 5$  and VpreB (Kitamura et al. 1992; Mundt et al. 2001) indicating that signaling from the pre-BCR is critical for the pro-B to pre-B cell transition.

Signaling from the pre-BCR shares several features with BCR signaling in mature B cells including a common signal transduction pathway (Guo et al. 2000). However, although antigen binding is the stimulus for BCR signaling, it is still unclear whether pre-BCR signaling is ligand-dependent. Studies using soluble pre-BCR indicated that that unlike BCR fragments, soluble pre-BCR readily bound to the surface of stromal cell

lines (Bradl and Jack 2001). Subsequent experiments revealed that the stromal cell specific lectin galectin-1 directly bound the pre-BCR and was capable of initiating downstream tyrosine phosphorylation in pre-B cell lines (Gauthier et al. 2002), suggesting that interactions with stromal cells serve to activate pre-BCR signaling. Despite these associations, pre-B cells were found capable of proliferation and differentiation *in vitro* in the absence of supporting stromal cells (Rolink et al. 2000). Additionally, Monroe and colleagues observed that forced expression of Ig $\alpha$  and Ig $\beta$  alone was sufficient to inhibit IgH rearrangement, stimulate proliferation, and promote IgL recombination in pro-B cells, suggesting that pre-BCR signaling is ligand-independent (Fuentes-Panana et al. 2004). Ohnishi and Melchers demonstrated that an arginine rich portion of  $\lambda 5$  is required for efficient pre-BCR signaling and proposed that ligand-independent signaling is achieved via  $\lambda 5$ -mediated pre-BCR internalization (Ohnishi and Melchers 2003), however, more recent data suggests that  $\lambda 5$  may in fact confer polyreactivity that is responsible for pre-BCR activity (Kohler et al. 2008).

Aggregation of pre-BCRs at the cell surface is thought to promote signaling by bringing several downstream signal transducing factors into proximity of one another (Fig. 1-2). One of the earliest factors to be recruited is the protein tyrosine kinase Syk (Spleen tyrosine kinase). Syk, along with Src family kinases, such as Lyn, phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of Ig $\alpha$  and Ig $\beta$  to which it is then able to bind via its Src homology 2 (SH2) domains. This association increases the kinase activity of Syk and promotes its autophosphorylation (Rowley et al. 1995). Syk plays a significant role in driving the proliferation of pre-B cells as evidenced by the phenotype of Syk<sup>-/-</sup> B cells, which are developmentally arrested at the pre-B cell stage (Cheng et al. 1995; Turner et al. 1995). Once activated, Syk phosphorylates the B cell co-receptor CD19, which in turn leads to the recruitment and activation of phosphoinositide 3 kinase (PI3K) (Aiba et al. 2008). PI3K acts as part of a pathway that

regulates several biological processes including cell proliferation. When activated PI3K phosphorylates the 3 position hydroxyl group of the inositol ring of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) located in the plasma membrane. The resulting molecule, phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>), acts as a second messenger that recruits additional proteins to the plasma membrane via interactions with pleckstrin-homology domains. Two such proteins are 3-phosphoinositide-dependent protein kinase 1 (PDK1) and its target Akt, which is a primary regulator of cell proliferation (reviewed in (Manning and Cantley 2007)). Thus, pre-BCR signaling promotes cell proliferation via the PI3K-PDK1-Akt pathway.

In addition to driving proliferation, the PI3K-Akt signaling pathway also appears to be involved in inhibiting IgL rearrangement. Clearly, preventing gene rearrangement during periods of multiple cell divisions is beneficial as it ensures genomic stability and prevents possible malignant transformation. PI3K-Akt signaling mediates suppression of IgL rearrangement through interactions with the FOX class O family of transcription factors. There are four members of this subgroup of transcription factors, Foxo1, Foxo3a, Foxo4, and Foxo6, all of which share the forkhead box DNA binding domain (Coffer and Burgering 2004). Foxo3a is the dominant isoform expressed in lymphocytes and has been shown to regulate lymphocyte homeostasis and T cell tolerance (Lin et al. 2004). Foxo proteins are direct targets of Akt and when phosphorylated are removed from the nucleus and degraded (Biggs et al. 1999; Takaishi et al. 1999). Recent studies by the Schlissel lab have revealed that Foxo1 is capable of activating transcription of Rag1 and Rag2 and that Akt activity decreased Rag levels in both pro-B and immature B cells (Amin and Schlissel 2008). Similarly, experiments by Jumaa and co-workers revealed that Foxo3a activity increased Ig $\kappa$  expression in pre-B cells and that this effect could be blocked by Akt-mediated phosphorylation of Foxo3a (Herzog et al. 2008).

Taken together these data demonstrate the important role of the PI3K–Akt pathway in the regulation of pre–B cell development.

Pre–BCR signaling is also required for initiation of IgL rearrangement (Reth et al. 1987). Pre–BCR regulation of IgL rearrangement appears to be highly dependent upon SLP65 (also known as BLNK and BASH), which may play a key role in separating proliferative and differentiative pre–BCR signals. Syk–mediated phosphorylation of SLP65 facilitates binding of additional signaling factors to SLP65, such as Bruton’s tyrosine kinase (Btk) and phospholipase C $\gamma$ 2 (PLC $\gamma$ 2), and promotes its function as a scaffold protein for construction of a multimeric signalosome (Koretzky et al. 2006). This complex has been shown to be important in regulating in IgL rearrangement both by downregulating pre–BCR expression and actively promoting Ig $\kappa$  rearrangement. Recent studies have found that the Ikaros–family transcription factor Aiolos downregulates  $\lambda$ 5 expression through interaction with its promoter (Thompson et al. 2007). Importantly, expression of Aiolos in pre–B cells is dependent upon the presence of SLP65, suggesting that pre–BCR signaling downregulates pre–BCR surface expression through a negative–feedback loop. SLP65 has also been implicated in activating Ig $\kappa$  gene rearrangement as pre–B cells from SLP65 mutant mice displayed reduced Ig $\kappa$  germline transcription and rearrangement (Hayashi et al. 2003). Interestingly, SLP65 mutant mice that also express a mutated form of the membrane co–receptor CD19 (**Fig. 1–2**) experienced greater reductions in both measures, suggesting that multiple pathways downstream of the pre–BCR likely regulate IgL recombination. SLP65 also appears to promote Ig $\kappa$  rearrangement through inhibition of Akt, thereby promoting Foxo activity (Herzog et al. 2008), as well as via regulation of interferon–regulatory factor 4 (IRF–4) (Thompson et al. 2007), a critical B cell regulatory factor (see below).

Downstream of the pre–BCR, several additional factors contribute to the regulation of IgL recombination. Two targets for the control of Ig $\kappa$  transcription and

rearrangement are enhancers elements located within the locus (**Fig. 1–3**). The murine Ig $\kappa$  locus is comprised of roughly 120 V $\kappa$  gene segments located at the 5' end of the locus and spanning a distance of approximately 30Mb (Roschenthaler et al. 1999). 20kb downstream of the V $\kappa$  gene segments are 5 J $\kappa$  gene segments, of which 4 are functional (J $\kappa$ 3 is a pseudogene (Max et al. 1979)), followed by the  $\kappa$ -constant region (C $\kappa$ ) which is located 2.5kb downstream of J $\kappa$ 5 (Kirschbaum et al. 1998; Kirschbaum et al. 1999). One of the Ig $\kappa$  enhancers, iE $\kappa$ , is located in the intron between J $\kappa$  and C $\kappa$  (Picard and Schaffner 1984), while the second, 3'E $\kappa$ , is located 9kb downstream of C $\kappa$  (Meyer and Neuberger 1989). Deletion of these enhancers revealed their critical role in driving Ig $\kappa$  rearrangement. Deletion of either iE $\kappa$  (Takeda et al. 1993; Xu et al. 1996) or 3'E $\kappa$  (Gorman et al. 1996) resulted in decreased Ig $\kappa$  rearrangement and fewer Ig $\kappa$  expressing B cells, while simultaneous deletion of both enhancers resulted in a complete block in Ig $\kappa$  rearrangement, indicating that the enhancers have synergistic effect (Inlay et al. 2002).

Although several transcription factor binding sites have been identified in the enhancers, to date E2A is the only transcription factor whose binding has been shown to be critical for Ig $\kappa$  rearrangement, as mutation of E2A binding sites in iE $\kappa$  reduced Ig $\kappa$  rearrangement to a similar degree as iE $\kappa$  deletion (Inlay et al. 2004). In addition to E2A, the transcription factors IRF-4 and IRF-8 activate and promote Ig $\kappa$  rearrangement. These two transcription factors are highly homologous to one another and are expressed exclusively in lymphocytes (Tamura et al. 2008). Both IRF-4 and 8, when complexed with the Ets-family members PU.1 or Spi-B, bind to an Ets-IRF composite element within the 3'E $\kappa$ , suggesting a role in regulating Ig $\kappa$  activity (Eisenbeis et al. 1995). Indeed, IRF-4/8 double mutant mice demonstrated a block in B cell development at the pre-B cell stage, with a loss of IgM<sup>+</sup> cells and deficient Ig $\kappa$  germline transcription and rearrangement (Lu et al. 2003). Interestingly, either IRF-4 or IRF-8 alone was

sufficient to rescue development and promote Ig $\kappa$  rearrangement in double mutant mice, indicating their functional redundancy (Ma et al. 2006). Additionally, IRF-4, together with Spi-B, have been shown to be sufficient to promote germline transcription of the Ig $\kappa$  locus in Abelson murine leukemia virus-transformed pro-B cell lines (Muljo and Schlissel 2003).

The mechanisms for IRF-4,8 mediated regulation of IgL rearrangement are still being characterized, however, Singh and colleagues recently reported that IRF-4 may influence Ig $\kappa$  recombination by two independent pathways (Johnson et al. 2008). First, IRF-4 was observed to promote IgL recombination by positioning Ig $\kappa$  alleles away from centromeric heterochromatin, which presumably increases Rag accessibility (Johnson et al. 2008). Additionally, IRF-4 was found to influence interleukin 7 (IL-7) signaling in pre-B cells. The IL-7 receptor (IL-7R) is required for B cell development, as IL-7R deficient mice display arrested development at the pro-B cell stage (Peschon et al. 1994). Specifically, it is thought that IL-7 signaling promotes survival of pro-B cells and proliferation of early pre-B cells. Importantly, after proliferation, late pre-B cells are less responsive to IL-7 which promotes IgL rearrangement by upregulation of Rag (Marshall et al. 1998; Johnson et al. 2008). IRF-4 may be involved in this decreased responsiveness by facilitating migration of pre-B cells away from IL-7 expressing stromal cells in the bone marrow. IRF-4 induced expression of the chemokine receptor CXCR4 in pre-B cells, which is significant since CXCL12, the ligand of CXCR4, is expressed by a small group of bone marrow stromal cells that are separated from those that express IL-7 (Tokoyoda et al. 2004), and suggests that IRF-4 mediated expression of CXCR4 results in the migration of pre-B cells away from IL-7. Furthermore, decreased IL-7 signaling resulted in enhanced binding of E2A to the E $\kappa$ i which, along with the finding that recruitment of E2A to 3'E $\kappa$  is dependent on IRF-4 (Lazorchak et al. 2006), indicates that IRF-4 also enhances IgL rearrangement through indirect activation

of Ig $\kappa$  enhancers. Together these findings reveal that IRF-4 orchestrates several discrete pathways in order to drive the development of pre-B cells and promote IgL rearrangement.

### **Mechanisms of Immunological Tolerance**

The generation and expression of surface IgM in immature B cells represents another critical developmental checkpoint and is also the first point at which BCR specificity can be assessed. As discussed earlier, BCR diversity is generated through the recombination of Ig gene segments at both the heavy and light chain loci. Along with the random association of IgH and IgL chains, this combinatorial diversity provides the potential to produce over  $1.9 \times 10^6$  unique receptors (Janeway et al. 2008). Due to the random nature of the process, however, it is also possible to generate BCRs that recognize host or self-antigens. Indeed, several studies suggest that a high proportion of newly generated BCRs are autoreactive. Through random IgH and IgL pairing studies in Ig transgenic mice thought to mimic IgH/IgL pairing *in vivo*, Novobrantseva *et al.* found that 9 out of 18 BCRs tested showed signs of autoreactivity (Novobrantseva et al. 2005). Unfortunately, the authors did not confirm BCR autoreactivity directly, instead choosing to measure pre-B cell compartment expansion and endogenous IgL rearrangement, both of which are indicative of receptor editing, a process of continued IgL rearrangement associated with autoreactivity (see below).

More conclusive evidence for immature B cell autoreactivity came from studies by the Nussenzweig lab. In these experiments IgH and IgL chains were cloned from single immature and mature human B cells and tested for autoreactivity (Wardemann et al. 2003). The authors found that compared to mature cells, IgH chains from immature B cells possessed longer complementarity determining regions 3 (CDR3s) which is a feature associated with autoreactive BCRs (Ichiyoshi and Casali 1994; Crouzier et al.



1995; Klonowski et al. 1999). Additionally, when the antibodies were tested for reactivity to cellular antigens, antibodies from immature cells were found to be highly autoreactive with nearly 76% displaying high levels of reactivity in a HEp2 ELISA. Furthermore, 55% of the immature B cell derived antibodies were also found to be polyreactive, as they demonstrated high degrees of reactivity to single- and double-stranded DNA (ssDNA and dsDNA), insulin, and the bacterial antigen LPS. Interestingly, the frequency of autoreactive B cells gradually declined as the B cells matured, as fewer antibodies from early emigrant peripheral (transitional) B cells were found to bind autoantigens and even fewer from fully mature cells. This finding suggests that although autoreactive B cells may be plentiful in the immature pool, they are likely altered or removed so that the mature B cell population is mostly non-autoreactive. Consistent with this idea, a higher frequency of autoreactive B cells were found among murine transitional B cells than mature follicular B cells (Merrell et al. 2006).

If allowed to fully mature and participate in immune responses, autoreactive B cells could mediate host cell damage and promote autoimmune disease. Consequently, several self-tolerance mechanisms exist to restrict the development or activation of autoreactive B cells. Studies using BCR transgenic mice have revealed three major tolerance mechanisms that act to influence the fate of autoreactive B cells, namely clonal deletion, anergy, and receptor editing. Clonal deletion refers to the elimination of autoreactive cells, while anergy refers a non-responsive state that lymphocytes can adopt such that they no longer respond to their cognate antigens. Receptor editing is the process whereby B cells with a functional BCR continue Ig rearrangement (primarily at the IgL locus) in order to alter BCR specificity. The system that controls which tolerance mechanism is engaged in autoreactive B cells is not fully understood, however, BCR affinity for self-antigen appears to be important. Goodnow and colleagues found that autoreactive B cells were anergized when presented with a soluble self-antigen,

however, when the same antigen was expressed as a membrane protein, the self-reactive B cells underwent clonal deletion (Hartley et al. 1991). Since membrane bound proteins are likely to interact with B cells in multivalent structures, it was proposed that increased BCR signaling caused deletion, while inefficient signaling by soluble molecules resulted in anergy. Consistent with this model, Fulcher *et al.* found that transferred autoreactive B cells persisted longer in the spleens of mice expressing a soluble form of autoantigen compared to those expressing a membrane-bound form (Fulcher et al. 1996).

The studies by Nemazee and Bürki were the first to conclusively demonstrate clonal deletion as a mechanism of tolerance (Nemazee and Burki 1989). In these experiments transgenic mice were generated that produced B cells expressing an IgM specific for H-2<sup>k</sup> MHC class I haplotype (3-83 idiotype (Id)). These mice were then bred onto backgrounds with differing MHC class I haplotypes. In mice expressing the MHC class I H-2<sup>d</sup> haplotype, 3-83Id<sup>+</sup> B cells were frequent, comprising up to 50% of splenic B cells. However, in H-2<sup>d/k</sup> mice, which also express the H-2<sup>k</sup> haplotype, no transgene expressing B cells were found. Intriguingly, H-2<sup>d/k</sup> mice still possessed splenic IgM<sup>+</sup> B cells, however, the total number was decreased by approximately 50%, suggesting that the 3-83Id<sup>+</sup> cells were selectively removed from the repertoire. These findings were confirmed in experiments by the Goodnow lab using a separate transgenic model (Hartley et al. 1993). In double transgenic mice (anti-HEL/mHEL mice), which express hen egg lysozyme (HEL) ubiquitously on the cell surface and also generate anti-HEL B cells, the authors found that the autoreactive cells died in 1 to 3 days. Interestingly, they observed that death of these cells was preceded by developmental arrest, which could be reversed with the removal of HEL. Additionally, they were able to prolong the survival of anti-HEL B cells through the forced expression of the anti-apoptosis gene bcl-2, suggesting that deletion of the cells occurred via the apoptotic pathway. In

subsequent studies, Kuramari *et al.* induced apoptosis *in vivo* through the introduction of self-antigen to anti-autoreactive B cells. These B cells expressed BCRs specific for red blood cells (RBCs) and had escaped tolerance by migrating to the peritoneum. However, upon injection of RBCs into the peritoneum the autoreactive cells efficiently underwent apoptosis, as measured by DNA fragmentation (Murakami *et al.* 1992).

Anergy is an alternative fate for B cells that recognize self-antigen. The studies of anti-HEL transgenic mice by Goodnow and colleagues in 1988 were the first to describe this phenotype (Goodnow *et al.* 1988). They observed that instead of being deleted, when anti-HEL-reactive B cells developed in the presence of secreted HEL they entered a state of unresponsiveness marked by loss of IgM secretion and decreased IgM (but normal IgD) surface expression. In contrast, Erikson *et al.* observed in the anti-DNA heavy chain transgenic model 3H9, that although autoreactive cells were anergized, as determined by low serum IgM levels, the surface IgM expression of these cells was unchanged. This finding suggests that decreased IgM expression is not always indicative of anergic cells. Additionally, Clarke and co-workers found that anergic B cells in an anti-Sm transgenic model displayed an immature B cell phenotype, expressing high levels of heat-stable antigen (HSA) and lacking expression of CD23 (Santulli-Marotto *et al.* 1998). Thus, these reports demonstrate that anergic B cells in autoreactive BCR transgenic models can adopt variable phenotypes. To address this problem Merrell *et al.* sought to characterize anergic cells in the context of a wild-type B cell repertoire. Using two different transgenic models of anergy, they defined anergic cells as having a T3-like phenotype (AA4.1+, CD23+, IgM<sup>low</sup>) and also found that they expressed high levels of IgD and levels of HSA intermediate between T2 and mature follicular cells (Merrell *et al.* 2006). Furthermore, these T3-like cells were not found in transgenic models known to lack anergic B cells. Importantly, using this phenotype the authors were able to identify anergic B cells from wild-type mice. This group of cells

did not proliferate or secrete IgM in response BCR stimulation and was found to be enriched for autoreactive cells. The molecular mechanisms responsible for the anergic phenotype appear to be related to altered signaling, as the cells demonstrate impaired tyrosine phosphorylation after BCR aggregation (Cooke et al. 1994) and have altered intracellular  $\text{Ca}^{2+}$  concentrations, which are elevated basally and do not increase after BCR stimulation (Benschop et al. 2001).

Increasing evidence indicates that receptor editing plays a major role in the regulation of self-tolerance. This process involves the continued rearrangement of Ig genes, primarily at the IgL loci, in order to achieve an alteration in BCR specificity. The structure of the Ig $\kappa$  locus is ideally suited for editing rearrangements due to the presence of multiple J $\kappa$  gene segments (**Figure 1-4**). In B cells with existing V $\kappa$ J $\kappa$  rearrangements, upstream un-rearranged V $\kappa$  gene segments are able to recombine with downstream unused J $\kappa$  gene segments, thereby “leap-frogging” the existing rearrangement and producing a new IgL variable region with a new specificity. Additionally, in situations where all J $\kappa$  gene segments have been exhausted, V $\kappa$  genes (or an RSS in the J $\kappa$ -C $\kappa$  intron) can rearrange to the recombining sequence (RS, also known as the  $\kappa$  deleting element (KDE) in humans) (Durdik et al. 1984; Siminovitch et al. 1985). Rearrangement to RS, which is located approximately 25kb downstream of C $\kappa$ , results in functional inactivation of the Ig $\kappa$  allele through disruption (either deletion or inversion) of C $\kappa$ . Thus, RS recombination can promote rearrangement on the second Ig $\kappa$  allele or at the Ig $\lambda$  locus (Muller and Reth 1988). Indeed, it appears that IgL rearrangement occurs in an ordered manner with Ig $\kappa$  rearrangement occurring first, followed by RS recombination, and then Ig $\lambda$  rearrangement. Studies in mice with 2, 1, or no Ig $\kappa$  alleles revealed that delayed Ig $\lambda$  rearrangement occurs independently of Ig $\kappa$  rearrangement (Arakawa et al. 1996) and examination of rearrangement kinetics in pre-B cell lines revealed a hierarchy among IgL rearrangements (van der Burg et al. 2002;

Klein et al. 2005). These findings lead to the suggestion that RS recombination may serve to activate Ig $\lambda$  rearrangement directly through generation of a protein from the RS rearrangement, by activation of an Ig $\lambda$  enhancer, or by removing an Ig $\lambda$  suppressor (Persiani et al. 1987). However, studies of RS-derived RNA transcripts revealed that they were insufficient to induce Ig $\lambda$  rearrangement (Daitch et al. 1992). Furthermore, mice lacking the ability to rearrange RS were still capable of producing Ig $\lambda$ <sup>+</sup> B cells (Chen et al. 1993; Zou et al. 1993), suggesting that RS rearrangement is not a prerequisite for Ig $\lambda$  recombination. Despite these findings, in mice bearing a targeted deletion of RS, Nemazee and co-workers found a decrease in the number of Ig $\lambda$ <sup>+</sup> B cells indicating that although not required, RS recombination may promote development of Ig $\lambda$ -expressing B cells (Vela et al. 2008).

The process of receptor editing was first described in two separate studies by the Nemazee and Weigert labs. Using anti-H-2<sup>k,b</sup> transgenic mice (3-83Id), Tiegs *et al.* observed that when the membrane-bound autoantigens H-2<sup>k</sup> or H-2<sup>b</sup> were present, 3-83Id<sup>+</sup> B cells were removed from peripheral lymphoid organs, but could still be found in the bone marrow (Tiegs et al. 1993). Further analysis of 3-83Id<sup>+</sup> cells in the bone marrow revealed that they expressed increased levels of Rag-1 and Rag-2 mRNA compared to idiotype positive cells that had not encountered self-antigen. Additionally, Rag levels were further increased if the cells encountered a higher affinity autoantigen. Possible V(D)J recombination implied by increased Rag expression was confirmed by qPCR of excision products, which are DNA segments removed from the chromosome during Ig gene rearrangement. The benefit of these rearrangements was demonstrated by the visualization of 3-83Id<sup>+</sup> bone marrow cells that expressed endogenous Ig $\lambda$  and the finding that virtually all Ig $\lambda$ <sup>+</sup> peripheral B cells were also 3-83Id<sup>+</sup>. Similarly, the Weigert lab used a double IgH/IgL transgenic model to study the fate of anti-dsDNA B cells (Gay et al. 1993; Radic et al. 1993). They observed that among peripheral B cells

virtually all IgM<sup>+</sup> cells expressed the transgenic IgH, however, none of these cells retained expression of the transgenic IgL, suggesting that they had generated new light chains. Endogenous IgL rearrangement was confirmed by detection of Igκ gene rearrangement and mRNA transcripts in double-transgenic hybridomas. Interestingly, the light chain usage among peripheral cells was restricted to a few Vκ genes, suggesting that these light chains in particular were able to abrogate DNA binding and prevent cell death by clonal deletion. Consistent with this model, Radic *et al.* observed that hybridomas generated from splenocytes of single 3H9 IgH transgenic mice no longer bound dsDNA and expressed a limited set of light chains, indicating that only light chains able generate non-autoreactive BCRs with 3H9 survived and completed development (Radic et al. 1993). Additionally, these hybridomas demonstrated skewed usage of distal Jκ genes (Jκ5) which is indicative of continued Ig rearrangement and suggests that these cells were tolerized via receptor editing. Similar results were also observed in 3H9 transgenic mice that carried only one functional Igκ allele (Prak et al. 1994).

The overall contribution of receptor editing to B cell tolerance has been difficult to determine, however, recent data may provide more accurate estimates of editing frequencies. Calculating receptor editing levels in the transgenic models described above is unreliable due to the nature of the Ig transgenes used. Unlike normally expressed Ig chains which can be directly silenced through receptor editing rearrangements, the conventional transgenes employed in these models are not located in the endogenous loci and consequently cannot be removed through V(D)J recombination. Therefore, frequencies from these models may underestimate receptor editing as more B cells will undergo clonal deletion due to the inability to silence transgene expression. To address this issue Eline Luning Prak and Martin Weigert generated site-directed transgenic (sd-tg) mice in which the endogenous Jκ locus was

replaced with a functional V $\kappa$ 4J $\kappa$ 4 or V $\kappa$ 8J $\kappa$ 5 rearrangement (Prak and Weigert 1995). Because of the location of these transgenes and the retention of an endogenous downstream J $\kappa$ 5 gene segment in the V $\kappa$ 4 mice, B cells from these mice could silence the IgL transgenes via physiologic receptor editing rearrangements. Indeed, hybridomas generated from these mice revealed that additional IgL rearrangements took place not only on the endogenous allele, but on the transgenic allele as well. Additionally, these mice were bred with mice carrying a sd-tg for the 3H9 IgH (Chen et al. 1995), to create double sd-tg mice expressing anti-DNA antibodies (Chen et al. 1997). Analysis of hybridomas revealed that the mice did not harbor anti-DNA B cells and genetic studies demonstrated that this loss of autoreactivity was largely achieved by IgL rearrangement. Strikingly, 85% of hybridomas from 3H9/V $\kappa$ 4 mice had undergone IgL rearrangement on the targeted allele, while only 11% rearranged the endogenous allele without first inactivating the V $\kappa$ 4 transgene. Similar results were observed by the Rajewsky and Behrens labs in a separate sd-tg systems (anti-MHC class I and anti-HEL, respectively), where they found increased receptor editing levels that correlated with B cells expressing autoreactive BCRs (Pelandra et al. 1997; Hippen et al. 2005). Furthermore, using a system in which Ig $\kappa$  alleles can be distinguished by differences in C $\kappa$ , Nussenzweig and colleagues observed that approximately 25% of BCRs are generated via editing rearrangements (Casellas et al. 2001). Together these findings highlight the efficiency of receptor editing and suggest a role for receptor editing in tolerance induction in normal mice.

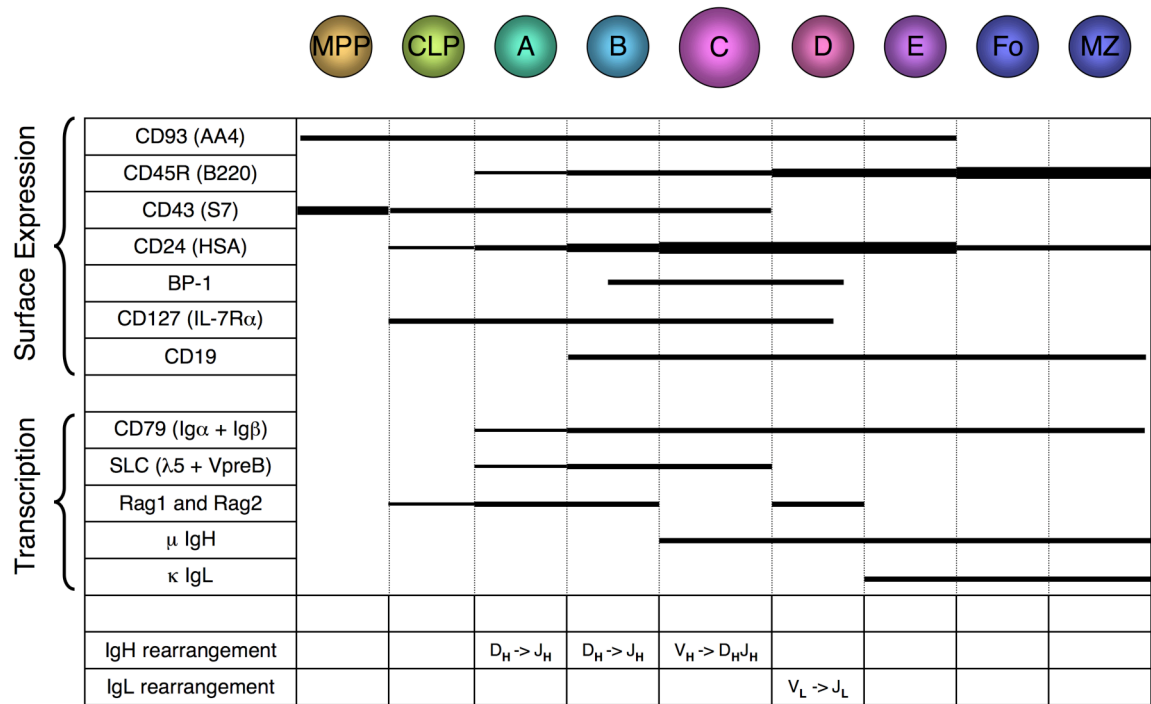
To date the most definite evidence demonstrating the significance of receptor editing in maintaining tolerance has come from studies of autoreactive sd-tg B cells in the context of a polyclonal repertoire (Halverson et al. 2004). In these studies Pelandra and co-workers generated chimeric mice that possess a subset of B cells expressing the 3-83Id (anti-H-2<sup>k,b</sup>) IgH and Ig $\kappa$  chains. The ratio of 3-83Id and wild-type cells mixed

varied in different chimeric mouse strains and provided a means of tracking the survival of the autoreactive B cells. Remarkably, transgene bearing B cells were not deleted and instead had replaced the transgenic Ig $\kappa$  chain with an endogenous Ig $\kappa$ , indicating that editing was highly efficient at rescuing these autoreactive cells from deletion. Importantly, when receptor editing was limited by removing free J $\kappa$  gene segments in the Ig $\kappa$  locus, up to 50% of the transgenic autoreactive cells underwent clonal deletion suggesting that receptor editing is the dominant form of central tolerance.

Clearly receptor editing is an important mechanism of B cell tolerance, however, our understanding of the role of editing in a wild-type repertoire has been hindered by the dependence on transgenic mouse models. Although these models can help us understand the causes and consequences of receptor editing, expression of transgenic Ig chains can complicate the interpretation of results due to alterations in B cell development. Moreover, they cannot tell us about the significance of editing in a wild-type polyclonal repertoire. Common markers of receptor editing, such as distal J $\kappa$  and increased Ig $\lambda$  usage that could be applied to wild-type mice, are confounded by clonal selection which can influence these measures. To address this problem, I developed a novel assay for measuring receptor editing rearrangements which does not rely upon surface BCR components that are subject to selection. Additionally, as editing may be important in preventing autoimmune disease, I examined mouse models of autoimmunity to determine if defects in receptor editing contribute to disease. These experiments were then translated to human patients with autoimmune disease. Finally, to investigate the forces that stimulate receptor editing, precursor rearrangements were analyzed in order to determine if editing is dependent upon self-antigen signaling. If this is the case, then functional precursors should be enriched for autoreactive IgL chains.

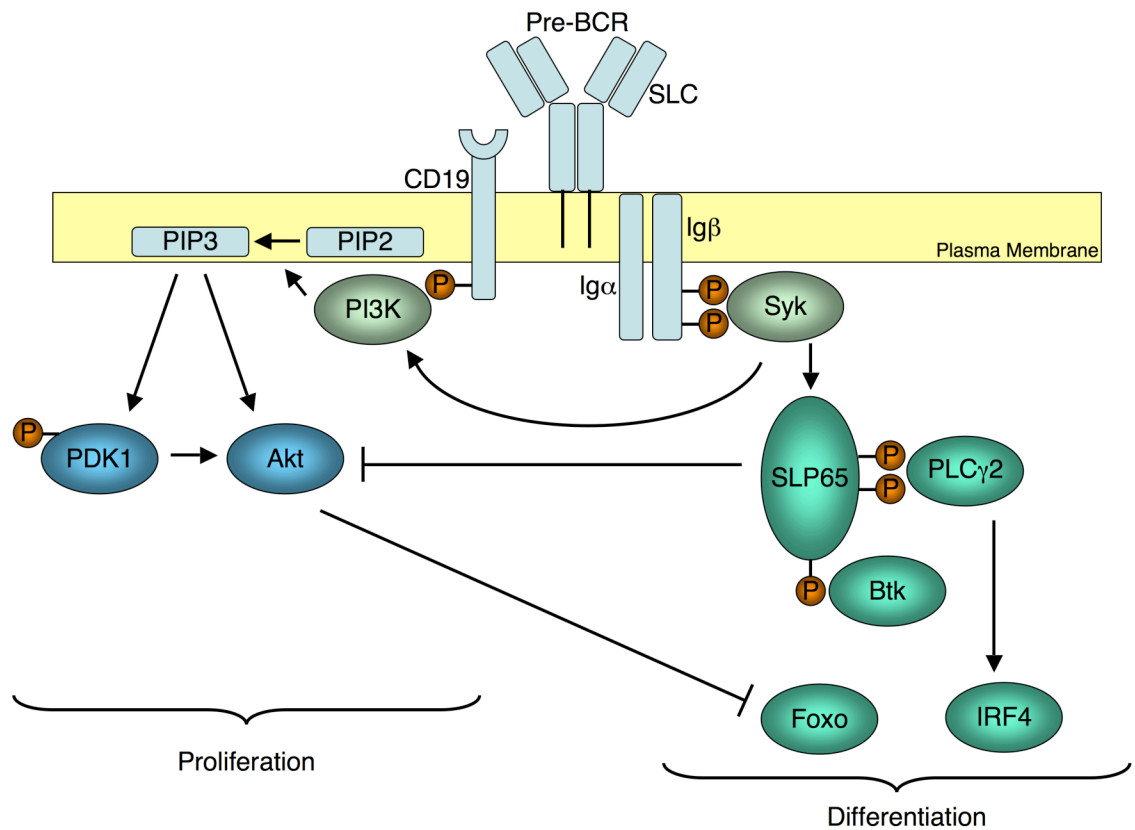


**Figure 1-1**



**Figure 1-1: B cell development is an ordered process involving progressive stages of maturation defined by changes in protein expression and gene transcription** (adapted from (Hardy et al. 2007)). Surface protein expression of different stages in B cell development are depicted in the first 7 rows. Gene transcription as determined by mRNA quantification or reporter constructs are depicted in the following 5 rows. The final two rows depict the status of ongoing immunoglobulin gene rearrangement during various stages of development. Darker lines depict increased expression. Letters designate bone marrow fractions as described in (Hardy et al. 1991). Abbreviations: MPP, multipotent progenitor; CLP, common lymphoid progenitor; Fo, follicular B cell; MZ, marginal zone B cell.

**Figure 1-2**



**Figure 1-2: Pre-B cell receptor signaling regulates pre-B cell proliferation and differentiation** (adapted from (Herzog et al. 2009)). The pre-B cell receptor (pre-BCR) is expressed at the pre-B cell stage and is comprised of two IgH chains that each pair with a surrogate light chain (SLC). The SLC itself is a complex of two proteins,  $\lambda 5$  and VpreB. The paired IgH and SLC represent the extracellular components of the pre-BCR that associate with Ig $\alpha$  and Ig $\beta$ , which serve to propagate signals within the cell. Ligation of the pre-BCR leads to recruitment of tyrosine kinases, such as Syk, that initiate the signaling cascade. Activation of phosphoinositide 3 kinase (PI3K) leads to activation of protein dependent kinase 1 (PDK1) which in turn activates Akt, a potent regulator of cellular proliferation. Additionally tyrosine kinases act upon SH2-domain

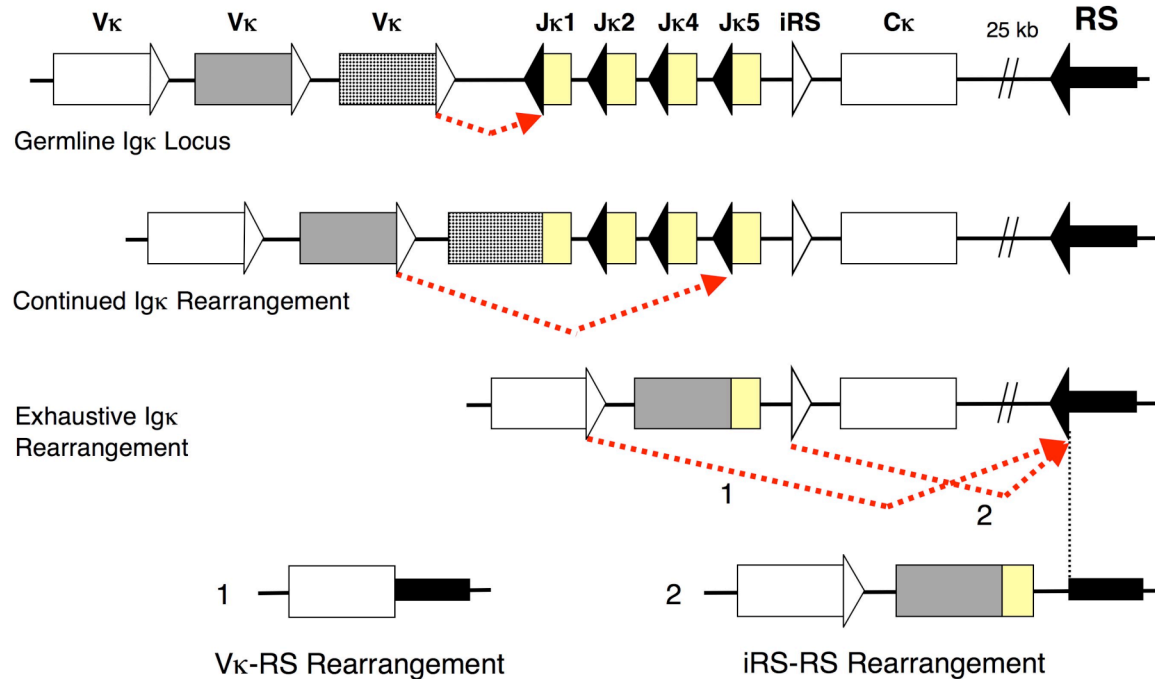
containing leukocyte protein of 65kDa (SLP65) which once phosphorylated can interact with additional signaling molecules, including Bruton's tyrosine kinase (Btk) and phospholipase C $\gamma$ 2 (PLC $\gamma$ 2). Recently SLP65 has been shown to block Akt inhibition of the transcription factor Foxo1 (Herzog et al. 2008), while PLC $\gamma$ 2 has been shown to upregulate expression of interferon regulatory factor-4 (IRF-4) (Bai et al. 2007), suggesting that this arm of pre-BCR signaling is important for pre-B cell differentiation and immunoglobulin light chain rearrangement.

**Figure 1-3**



**Figure 1-3: Schematic of the germline murine Igκ locus** (adapted from (Schlissel 2004)). The germline Igκ locus is displayed with exons depicted as boxes and introns as dark lines. Recombination signal sequences (RSSs) are depicted as triangles with open triangles representing 12bp RSS spacers and filled triangles are 23bp spacers. Ovals depict the two Igκ transcriptional enhancer elements. The recombining sequence (RS) is located approximately 25kb downstream of Cκ and can recombine with upstream Vκ gene segments or a cryptic heptamer located in the Jκ-Cκ intron (iRS).

**Figure 1-4**



**Figure 1-4: Receptor editing at the Igκ locus.** (a) Schematic of the murine Igκ light chain locus illustrating a primary Vκ-Jκ rearrangement followed by a “leap-frogging” editing rearrangement involving an upstream 5’ Vκ gene segment and a downstream unrearranged Jκ gene segment. In situations where free Jκ gene segments have been exhausted, editing can continue via RS rearrangement. Two pathways of RS rearrangement are available. The first (1) involves recombination of an upstream unrearranged Vκ gene segment to RS, while the second (2) utilizes a non-canonical recombination signal sequence (iRS) in the Jκ-Cκ intron to rearrange to RS. Both result in the deletion of Cκ and functional inactivation of the Igκ locus. Exons are given as boxes, recombination signals are indicated by triangles and dashed lines with arrows illustrate the rearrangements.

## **CHAPTER 2: RS Rearrangement Frequency as a Marker of Receptor Editing in Lupus and Type 1 Diabetes**

Published as part of

*The Journal of Experimental Medicine* 2008, Dec. 22, 205(13): 2985–94.

## INTRODUCTION

B cells undergo a random process of V(D)J recombination in order to generate the many distinct receptors needed to recognize a vast array of antigens. An inevitable consequence of this random process is the production of autoreactive B cells (Wardemann et al. 2003). An important mechanism for tolerizing autoreactive B cells is receptor editing (Halverson et al. 2004). Receptor editing results in the alteration of B cell receptor specificity and is achieved by ongoing immunoglobulin (Ig) gene rearrangement, most commonly at the light chain loci (Gay et al. 1993; Radic et al. 1993; Tiegs et al. 1993). Light chain rearrangement proceeds in an ordered fashion as B cells develop in the bone marrow, with  $\kappa$  genes recombining first, followed by rearrangement of the Recombining Sequence and  $\lambda$  (Lewis et al. 1982; Muller and Reth 1988). The Recombining Sequence (known as the Kappa Deleting Element (KDE) in humans, hereafter RS) is a non-coding gene segment located 25kb downstream of  $C\kappa$  in the  $\kappa$  locus that is rearranged during continued Ig light chain gene rearrangement (Durdik et al. 1984; Siminovitch et al. 1985).

Due to the unique structure of the  $\kappa$  locus, primary  $V\kappa$ - $J\kappa$  rearrangements that are non-functional or autoreactive can be replaced via “leap-frogging” recombination of un-rearranged upstream  $V\kappa$ - and downstream  $J\kappa$ -gene segments to form new  $\kappa$  light chains (**Fig. 2-1a**). Additional rearrangement attempts can be made through recombination at the second  $\kappa$  allele or at  $\lambda$ . Recombination of RS to upstream  $V\kappa$ -gene segments or a recombination signal sequence (RSS) within the  $J\kappa$ - $C\kappa$  intron results in the deletion or inversion of  $C\kappa$  and functional inactivation the  $\kappa$  locus (**Fig. 2-1a**). Because RS rearrangements do not encode any functional proteins (Daitch et al. 1992), monitoring RS rearrangement provides a specificity-independent means of measuring repeated rearrangement attempts at  $\kappa$  (receptor editing).

The original studies characterizing RS recombination postulated that it served to promote  $\lambda$  rearrangement by either repressing  $\kappa$  rearrangement or activating the  $\lambda$  locus (Persiani et al. 1987; Muller and Reth 1988). However,  $\lambda$ -expressing B cells can form without undergoing RS rearrangement, indicating that RS is not required for the production of  $\lambda$  (Zou et al. 1993). When RS rearrangement is prevented in RS knock-out mice, receptor editing is inefficient and autoreactive B cells are found among peripheral cells (Vela et al. 2008), highlighting the potential role of RS in establishing central tolerance and reducing light chain allelic and isotypic inclusion.

Current clinical assays that evaluate B lymphocyte tolerance focus on serum autoantibodies, which are products of mature B cells. Because secreted autoantibodies are an end-product rather than an intermediate, they do not distinguish between autoimmunity that arose during primary B cell maturation or later due to events such as somatic mutation. The distinction is important because a defect in primary B cell tolerance may predict disease development. Furthermore, diseases occurring as a result of a primary B cell tolerance defect may be associated with resistance to B cell targeted therapy because the primary repertoire is predicted to rapidly repopulate with autoreactive cells if B cell reconstitution is allowed to proceed. Before testing those ideas, an assay for central B cell tolerance is needed.

This manuscript describes the development and initial characterization of RS rearrangement frequency as an assay for central B cell tolerance in systemic lupus erythematosus (SLE) and type 1 diabetes (T1D). In both of these diseases B cells play a critical pathogenic role. Autoantibodies are a prominent feature, whether they be directed against nuclear antigens (in SLE) (Tan 1982) or pancreatic  $\beta$ -cell antigens such as GAD65 or insulin (in T1D) (Leslie and Delli Castelli 2004). Furthermore, B cell deficiency or depletion in mouse models ameliorates or prevents disease (Shlomchik et al. 1994; Serreze et al. 1996; Noorchashm et al. 1997; Ahuja et al. 2007; Hu et al.



2007). The results with B cell depletion therapy in humans are equivocal for SLE (EXPLORER study – ClinicalTrials.gov identifier: NCT00137969) and not yet fully explored in T1D. Here we describe the status of central tolerance in individuals with SLE or T1D using RS rearrangement frequency as a marker.

## RESULTS

### A novel assay for estimating levels of receptor editing

We focused on RS rearrangement as an assay for receptor editing because RS rearrangement is known to accompany extensive light chain rearrangement during primary B cell maturation (Persiani et al. 1987; Klein et al. 2005) and the major RS rearrangement products are defined and share a common DNA sequence (Siminovitch et al. 1987). Most importantly, RS rearrangements do not encode a functional protein (Daitch et al. 1992), and therefore are independent of antibody specificity, making their measurement potentially applicable to any disease in which B cells play a pathogenic role. By combining a quantitative PCR assay for RS rearrangement frequency with cell sorting, receptor editing can be analyzed in different B cell subsets (**Fig. 2-2**).

We analyzed the most abundant class of RS rearrangements, which are V $\kappa$  to RS in mice and iRS to RS in humans (Retter and Nemazee 1998; Brauninger et al. 2001). For the human studies, iRS to RS rearrangements were quantified relative to an absolute standard consisting of a cloned iRS–RS rearrangement that was serially diluted in fibroblast DNA. Log–linear amplification was observed over the range of 0.7–200% RS rearrangements per cell genome (**Fig. 2-3**). 200% corresponds to having two RS rearrangements, one on each  $\kappa$  allele. Typical RS frequency measurements fall within this log–linear range. For the mouse studies a degenerate V $\kappa$  primer was used for RS rearrangement measurements (Schlissel and Baltimore 1989). Murine V $\kappa$ –RS rearrangements were quantified as fold difference relative to IgM<sup>+</sup>,  $\kappa$ <sup>+</sup> spleen DNA from

B6 mice (the spleen contains a mixture of different  $V_{\kappa}$ s). Fold differences were used rather than absolute frequencies in order to avoid confounds caused by differing amplification efficiencies for different  $V_{\kappa}$  genes.

The correlation between RS rearrangement and extensive light chain rearrangement has been established in earlier work from several groups (Durdik et al. 1984; Moore et al. 1985; Siminovitch et al. 1985; Dunda and Corcos 1997; Retter and Nemazee 1998; Brauninger et al. 2001). Consistent with these earlier reports, RS rearrangement is increased 8-fold amongst  $\lambda^+$  murine IgM<sup>+</sup> B cells compared to  $\kappa^+$  cells (**Fig. 2-1b**). Amongst CD19<sup>+</sup> human B cells, 16% of  $\kappa^+$  B cells carried an RS rearrangement and the frequency of RS rearrangements amongst  $\lambda^+$  B cells was greater than 100%, indicating that some  $\lambda^+$  B cells have rearranged to RS on both  $\kappa$  alleles. Because RS levels are higher in  $\lambda^+$  than in  $\kappa^+$  B cells, the overall RS rearrangement frequency correlates inversely with the  $\kappa/\lambda$  ratio. To reduce the variability in RS frequency measurements introduced by the  $\kappa/\lambda$  ratio, RS analysis was performed separately in  $\kappa^+$  or  $\lambda^+$  cells. However, it is important to note that RS rearrangement levels in a given  $\kappa^+$  or  $\lambda^+$  B cell population vary independently from the overall  $\kappa/\lambda$  ratio (**Fig. 2-4** and **Fig. 2-9a**). Thus, measuring the RS frequency is not simply a cumbersome method for assessing the  $\kappa/\lambda$  ratio. Also of note, RS rearrangement frequencies are correlated in  $\kappa$  and  $\lambda$ -expressing B cells within single individuals. (This intra-individual correlation and the concept of an RS set point will be addressed in the analysis of human subjects below).

### **RS rearrangement levels vary between developmental and functional B cell subsets in mice**

If RS rearrangement is a marker of ongoing antibody light chain rearrangement, it should occur at the time of late  $\kappa$  or  $\lambda$  rearrangement. Consistent with this prediction,

the highest level of RS rearrangement was found among bone marrow fraction D cells (small pre-B II cells (Hardy et al. 1991), hereafter Fr. D), which are cytoplasmic Ig $\mu$ <sup>+</sup>, but no longer express surrogate light chain. Fraction D cells express the Rag1 and Rag2 proteins for light chain gene rearrangement (**Fig. 2-5a**) (Hardy et al. 1991). Further subsetting within Fr. D based upon BP-1 expression, revealed that the majority of RS rearrangements most likely occurred towards the end of this period (**Fig. 2-5b**).

In the subsequent developmental subset, Fr. E (newly formed IgM<sup>+</sup> immature B cells), V $\kappa$ -RS rearrangement levels were reduced by 3-fold and closer to those of mature, circulating B cells (Fr. F). The difference in V $\kappa$ -RS rearrangement levels between Fr. D cells and  $\kappa$ <sup>+</sup> Fr. E cells could be due to the inclusion of pre-B cells undergoing or completing  $\lambda$  rearrangement within Fr. D. However, RS levels in total IgM<sup>+</sup> Fr. E cells were similar to  $\kappa$ <sup>+</sup> Fr. E cells, suggesting that exclusion of  $\lambda$ <sup>+</sup> cells from Fr. E cannot fully account for the decrease. Death of RS<sup>+</sup> cells or their rapid exit from the bone marrow to a peripheral pool may contribute to the decrease in RS rearrangement levels in Fr. E relative to Fr. D.

#### **Mouse strains prone to autoimmunity display lower levels of RS rearrangement**

The preceding experiments established the developmental timing of RS rearrangement and documented a positive correlation between RS rearrangement frequency and an autoreactive antibody heavy chain in inbred mice. To determine if RS rearrangement is altered in the context of autoimmunity, we measured RS rearrangement frequency in two different mouse models of autoimmune disease, MRL/lpr mice as a model of SLE and non-obese diabetic mice (NOD) mice as a model of T1D. We observed decreases in RS rearrangement levels among Fr. D cells of both MRL/lpr (2.5-fold reduction,  $p < 0.05$ ) and NOD mice (3.7-fold reduction,  $p < 0.01$ ), when compared to C57Bl/6 mice (**Fig. 2-6a**). Interestingly, fewer RS rearrangements were

also observed among Fr. E cells in both MRL/lpr and NOD mice relative to C57Bl/6 mice. This decrease was evident in the mature recirculating B cells of the bone marrow (Fr. F) as well, implying that unedited or minimally edited cells persisted through development. Measurement of RS levels in splenic B cell subsets further substantiated this finding (**Fig. 2-6b**).

To determine if the lower levels of V $\kappa$ -RS rearrangements detected in MRL/lpr and NOD mice are attributable to strain effects, rather than as a consequence of an autoimmune state, RS levels were measured in MRL/MpJ mice, which share the same genetic background as MRL/lpr mice but lack the *Fas* mutation that is responsible for their lymphocytosis and accelerated systemic autoimmunity. Similarly, NOD mice were compared to NOR mice, which share the same diabetogenic MHC haplotype (H2<sup>g7</sup>) with NOD mice but are insulinitis-resistant and do not develop T1D (Prochazka et al. 1992) (**Fig. 2-7**). These "control" strains share the propensity to develop autoimmunity with MRL/lpr and NOD mice, but do so at a much slower pace. The control mice were analyzed at 3 months of age, a time at which autoimmune pathology is not yet apparent. Although RS rearrangement levels were slightly increased among some bone marrow B cell subsets in the control mouse strains, levels in mature B cells were comparable in MRL/MpJ and NOR mice to their more autoimmune counterparts. The low frequency of RS rearrangement in MRL/MpJ and NOR mice suggests that a reduced level of receptor editing predisposes towards the development of autoimmunity rather than arising as a consequence of autoimmune disease.

### **RS rearrangement levels are lower in human SLE and T1D**

To further investigate the level of receptor editing in the context of defective tolerance, we compared RS rearrangement levels in peripheral B cells from human subjects with established disease (SLE or T1D) to healthy control subjects (see Methods).

For this analysis, we evaluated 26 control subjects, 24 patients with SLE and 25 patients with T1D. Demographic features of the subject groups were compared and no significant differences were observed between control and SLE groups with respect to age, gender, and race; however the T1D group contained a larger proportion of men and Caucasians than the control and SLE groups (Table 2-1). Nonetheless, RS frequencies were not correlated with subject age, gender, or race (**Fig. 2-8**).

Amongst CD19<sup>+</sup>  $\kappa$ <sup>+</sup> B cells, RS rearrangement frequencies from SLE patients were lower on average compared to levels from healthy controls (**Fig. 2-9a**,  $p < 0.01$ ). The RS rearrangement frequency in  $\kappa$ <sup>+</sup> B cells from T1D was not significantly decreased compared to control subjects. In CD19<sup>+</sup>  $\lambda$ <sup>+</sup> B cells RS levels were lower in both SLE and T1D patients compared to control subjects (**Fig. 2-9b**,  $p < 0.01$ ). These results resemble those from the autoimmune mouse models and suggest that lower levels of RS rearrangement may be correlated with disease susceptibility.

We chose the bottom 10<sup>th</sup> percentile of the normal population as an arbitrary cut-off for the RS rearrangement frequency distribution in  $\kappa$ <sup>+</sup> B cells ( $\kappa$ RS). Using this cut-off, the fraction of subjects with a low  $\kappa$ RS value is 2/26 for the healthy control subjects, 13/24 for SLE and 8/25 for T1D. Using a similar  $\lambda$ RS cut-off, 3/26 controls, 9/23 SLE patients and 11/25 T1D patients had low RS levels. Additionally, RS levels were low in both  $\kappa$ <sup>+</sup> and  $\lambda$ <sup>+</sup> B cells in 7/23 SLE and 7/25 T1D patients, indicating that subjects with low  $\kappa$ RS levels tended to also have low  $\lambda$ RS levels. Indeed, Spearman correlations between the  $\kappa$ RS and  $\lambda$ RS measurements were significant in both healthy subjects and patients with autoimmune disease ( $r = 0.54$ ,  $p < 0.01$  and  $r = 0.52$ ,  $p < 0.01$ , respectively; **Fig. 2-9c**). Additionally, linear regression analysis revealed a lower degree of RS rearrangement in  $\lambda$ <sup>+</sup> cells relative to levels in  $\kappa$ <sup>+</sup> in patients with autoimmune disease compared to healthy controls. Together these findings suggest that some SLE and T1D patients have lower editing set points than those of most healthy individuals.

## DISCUSSION

The development of assays to monitor B cell tolerance and selection stringency can inform the choice of B cell targeted therapy for autoimmune disease. This contention is based upon the idea that individual patients with SLE and T1D have different tolerance defects (Florez et al. 2003; Harley et al. 2008). Previous assays that monitor B cell tolerance are problematic because they tend to measure relatively late events such as autoantibody production, rather than the pathways that B cells take on their way to becoming autoimmune. The RS rearrangement assay presented here takes two days to perform and provides insights into central (early) B cell tolerance. Furthermore, it can be combined with immunophenotyping to analyze editing levels in different peripheral B cell subsets. As such, it may be possible in the future to use this assay to monitor and stage defects in B cell tolerance.

The RS rearrangement assay gives an estimate of the overall level of light chain rearrangement in a defined population of B cells. The correlation between RS level and the light chain rearrangement level is based on earlier studies documenting that 10 to 15% of murine  $\kappa^+$  splenic B cells had undergone RS recombination, while virtually all  $\lambda^+$  splenic B cells harbored an RS rearrangement (Moore et al. 1985; Dunda and Corcos 1997; Retter and Nemazee 1998). Similarly, studies of single human B cells and acute lymphoblastoid leukemia cell lines demonstrated that cells expressing  $\lambda$  harbored more RS rearrangements than ones that expressed  $\kappa$  (Brauninger et al. 2001; van der Burg et al. 2002). Furthermore,  $\lambda$  B cells took approximately 24 hours longer to label with BrdU than B cells with  $\kappa$  rearrangements (Arakawa et al. 1996). Finally, studies in B cell leukemia cell lines subjected to Bcr-Abl tyrosine kinase blockade exhibited temporally ordered light chain rearrangements:  $\kappa$  followed by RS followed by  $\lambda$  (Klein et al. 2005). The current study is consistent with all of these previous studies: RS rearrangement

frequencies were higher in  $\lambda^+$  B cells than in  $\kappa^+$  B cells and were most frequent in late pre-B cells (Fr.D). Every other B cell subset, including Fr. E (which is where surface antibody is first detected) and more mature splenic B cells, exhibited *lower* RS levels. As RS rearrangements are non-revertible, these findings suggest that receptor editing fails to salvage some of the most highly edited B cells from clonal deletion.

The current study demonstrates decreases in RS rearrangement frequency in mouse models of SLE and T1D. Both MRL/lpr and NOD mice exhibited lower RS levels compared to wild-type C57Bl/6 mice. The finding of lower editing levels in MRL/lpr mice is consistent with recent work from the Feeney lab demonstrating decreased receptor editing in response to a ubiquitous membrane bound self antigen in transgenic MRL/lpr mice (Lamoureux et al. 2007). The finding of decreased RS rearrangement in NOD mice is at odds with a previous report in which editing was measured using a membrane bound facultative self-antigen (HEL) (Silveira et al. 2004). The RS assay, however, does not focus on one kind of self-antigen.

Although both MRL/lpr (Watanabe-Fukunaga et al. 1992) and NOD (Kishimoto and Sprent 2001) strains harbor previously characterized defects in apoptosis, lower RS levels were not linked these defects per se, as B cells from MRL/MpJ and NOR mice (both with intact apoptosis) also exhibited lower RS levels. In the case of MRL/lpr mice, the additional defect conferred by the *lpr* mutation (Watanabe-Fukunaga et al. 1992) may lead to less stringent selection of minimally edited immature B cells. Similarly, the finding of low RS rearrangement levels in both the NOD and NOR strains suggests that a second tolerance defect present only in NOD mice is required for development of diabetes. However, the genetic backgrounds of these control strains predispose them to develop autoimmunity. Despite having an intact *Fas* gene, MRL/MpJ mice spontaneously develop autoimmunity including pancreatitis (Kanno et al. 1992) and glomerulonephritis (Hewicker et al. 1990), but the disease is milder and occurs later in life. NOR/LtJ mice

share the diabetogenic H2<sup>g7</sup> haplotype with NOD mice and have altered macrophage and peripheral T cell compartments (Prochazka et al. 1992). All of the mice used for these experiments were 3 months of age, which is at a time that precedes disease development in NOR and MRL/MpJ mice. Together these findings suggest that decreased RS rearrangement levels in these mice reflect an altered propensity to develop autoimmunity.

In humans, lower RS rearrangement levels were also detected in both SLE and T1D patients compared to healthy control subjects. The decrease was most apparent in  $\lambda^+$  B cells, but the level of RS rearrangement was correlated in  $\kappa^+$  and  $\lambda^+$  B cells in individual patients. The levels were not proportional to the  $\kappa/\lambda$  ratio or to the fraction of B cells in the blood. These findings have theoretical and practical implications. The theoretical implication is that individuals may have different RS rearrangement "set points." The set points tend to be lower in T1D and SLE than in healthy subjects, but overlap to some degree. It will be important to directly measure RS levels in different peripheral B cell subsets and determine if differences can be correlated with B cell selection checkpoints or if, in a fashion similar to the mouse models, different subsets will show correlated RS levels. Up until now, selection checkpoints have been monitored by single cell antibody cloning and expression studies (Wardemann et al. 2003; Yurasov et al. 2005; Tsuiji et al. 2006). If RS rearrangement can be used instead, it may be possible to more easily define B cell tolerance checkpoints in individual patients with autoimmune disease. The practical implication of having correlated RS rearrangement frequencies in  $\kappa^+$  and  $\lambda^+$  B cells is that it may be possible to measure RS rearrangement frequency in whole blood rather than in sorted B cell subsets and then to correct the measurement for the B cell fraction and  $\kappa/\lambda$  ratio.

Given the concordance of RS rearrangement levels in T1D and SLE, we considered the possibility that upstream genetic defects could decrease receptor editing in both



disorders. One candidate is PTPN22 (protein tyrosine phosphatase non-receptor 22, also known as Lyp). PTPN22 is a lymphoid specific phosphatase that suppresses T cell activation (Cohen et al. 1999). A variant of PTPN22 (R620W) is common in both SLE and T1D (Bottini et al. 2004; Lee et al. 2007) and appears to have a more active phosphatase in the setting of T1D (Vang et al. 2005). Although the effects of PTPN22 R620W are not yet well defined in B cells (Rieck et al. 2007), one is tempted to speculate that there could be an increased BCR signaling threshold due to PTPN22 R620W that would result in decreased receptor editing. Other potential candidates include RAG or molecules that influence the intracellular localization or expression of RAG such as the nuclear importin KPNA1 (Glinsky 2008), interferon regulatory factor 4 (Johnson et al. 2008), or the transcription factor Foxo1 (Amin and Schlissel 2008; Herzog et al. 2008). Because RS rearrangement frequency corresponds to a phenotype rather than a heritable genetic alteration, there could be several different defects, each of which could contribute independently to a low RS frequency.

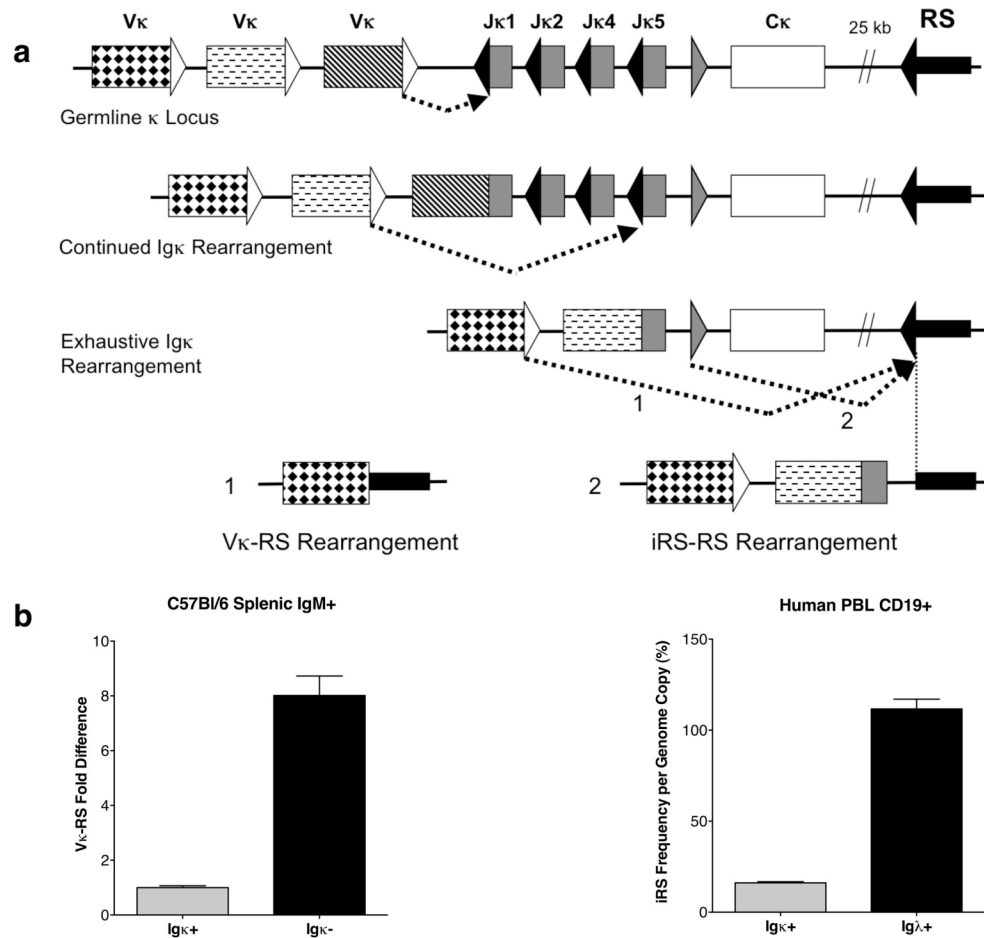
It is not yet clear what the biological consequences are of a low RS rearrangement frequency or if the lowest levels of RS rearrangement are inherently the most dangerous. Perhaps individuals with low RS levels (including a small subset of currently healthy subjects) are at increased risk of developing autoimmunity. In this regard, the prospective analysis of first-degree relatives of patients with autoimmunity might be especially informative. It will also be important to find out how generalizable the RS rearrangement frequency is to other autoimmune diseases as a potential marker for defective central tolerance.

## **ACKNOWLEDGEMENTS**

The authors thank L. Fisher (Division of Rheumatology) and C. Fuller (Division of Endocrinology) of the University of Pennsylvania School of Medicine for patient

recruitment, the research subjects for their participation in the study, the University of Pennsylvania Flow Cytometry and Cell Sorting Facility, Martin Sanders (Palingen, Inc.) for the 9G4 reagent, and Y. Louzoun (Bar Ilan University), Brad Johnson (University of Pennsylvania), and Martin Weigert (University of Chicago) for helpful discussions. This work was supported by the National Institutes of Health (U01DK070430 to A.N., E.L.P. and M.R.R.), University of Pennsylvania Institute for Diabetes, Obesity, and Metabolism, Alliance for Lupus Research, Lupus Foundation of Southern New Jersey, and the Lupus Foundation of America, Philadelphia Tri-State Chapter, Inc. The authors have no conflicting financial interests.

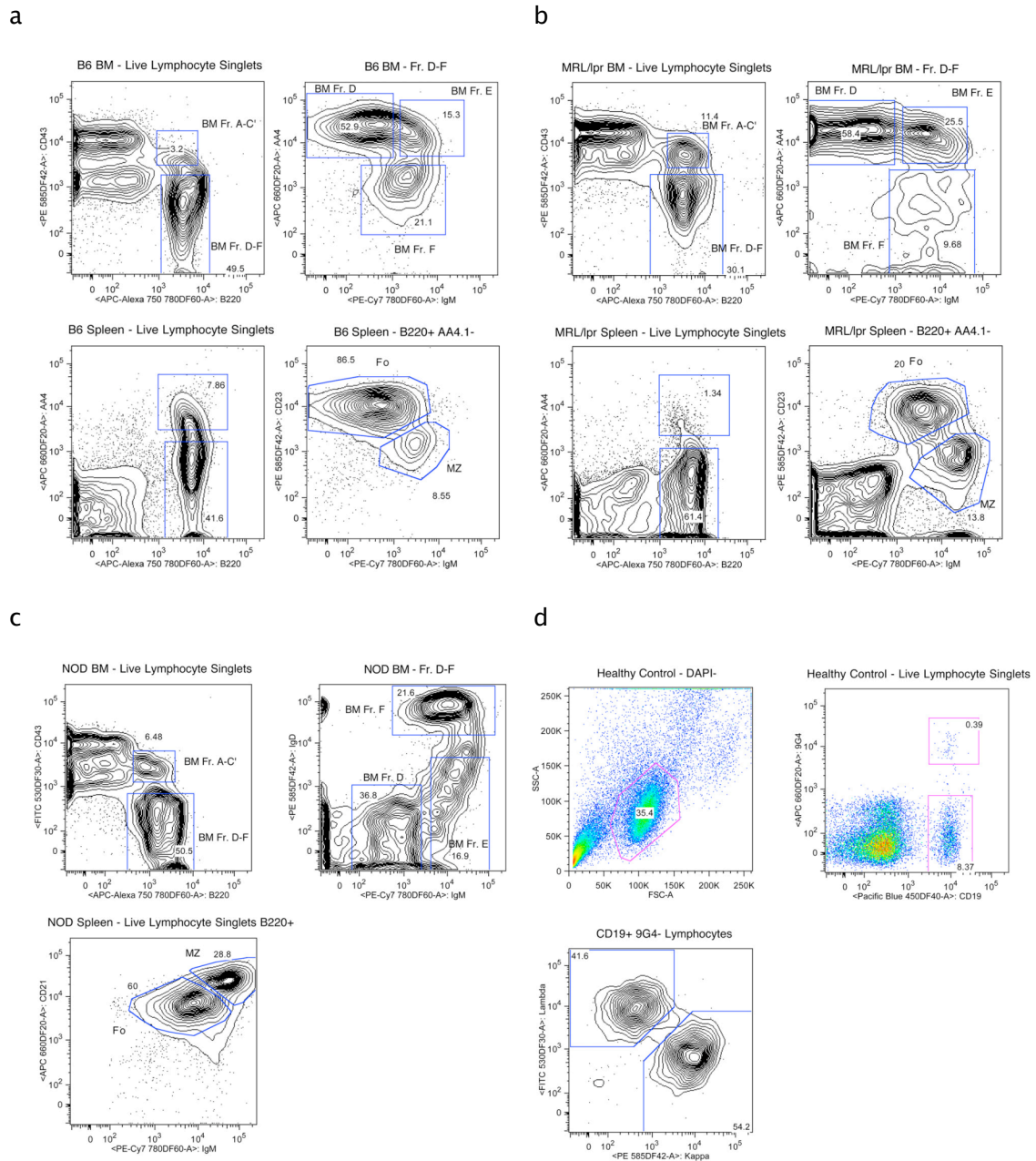
**Figure 2-1**



**Figure 2-1: RS rearrangement is a marker of extensive  $\kappa$  light chain rearrangement.** (a) Schematic of the murine  $\kappa$  light chain locus illustrating successive  $\text{V}\kappa$ - $\text{J}\kappa$  rearrangements followed by RS rearrangement. Two pathways of RS rearrangement are available. The first (1) involves recombination of an upstream unrearranged  $\text{V}\kappa$  gene segment to RS, while the second (2) utilizes a non-canonical recombination signal sequence (iRS) in the  $\text{J}\kappa$ - $\text{C}\kappa$  intron to rearrange to RS. Both result in the deletion of  $\text{C}\kappa$  and functional inactivation of the  $\text{Ig}\kappa$  locus. Exons are given as boxes, recombination signals are indicated by triangles and dashed lines with arrows

illustrate the rearrangements. **(b)** RS rearrangement levels as measured in Ig $\kappa$ <sup>+</sup> (gray bars) and Ig $\kappa$ <sup>-</sup> (presumed to be I+, black bars) from splenic B220<sup>+</sup> IgM<sup>+</sup> B cells of adult (3–4 month old) C57Bl/6 mice (n=5) and CD19<sup>+</sup> 9G4<sup>-</sup> peripheral B cells from healthy control subjects (n=26). All PCR reactions were performed in duplicate. Murine data are presented as fold difference relative to the average RS level in C57Bl/6 splenic B220<sup>+</sup> IgM<sup>+</sup> Ig $\kappa$ <sup>+</sup> B cells (+s.e.m.). Human data are depicted as rearrangement frequency per genome copy (+s.e.m.).

**Figure 2-2**

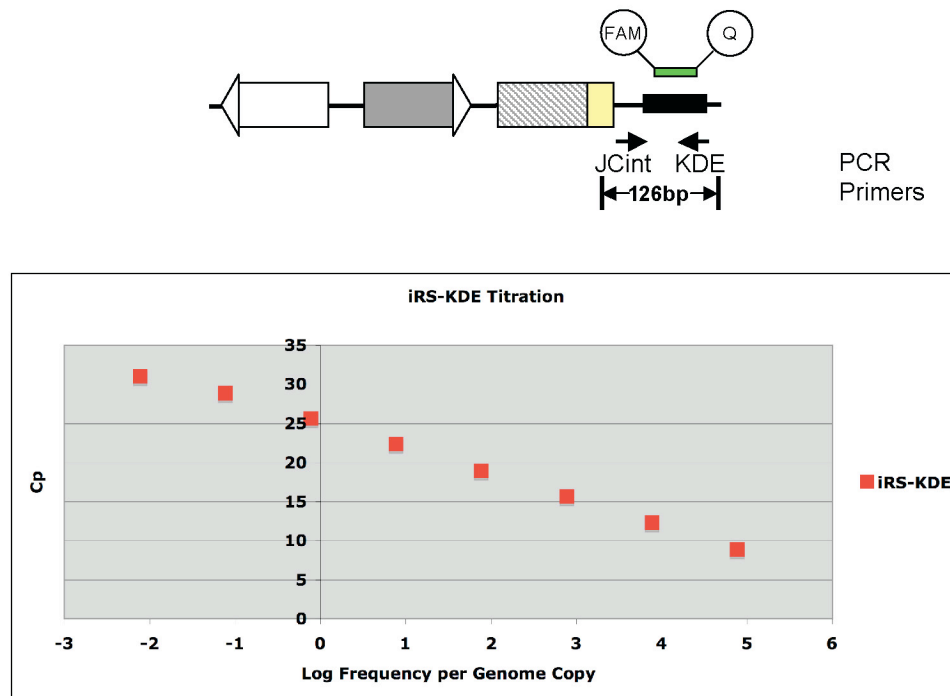


**Figure 2-2: Flow Cytometric Analysis of Murine and Human Lymphocytes.**

Cytometric analysis of bone marrow and splenic B cells from (a) C57Bl/6, (b) MRL/lpr, (c) NOD mice, and (d) healthy human subjects. Plots represent 50,000 events followed by DAPI<sup>+</sup> and doublet exclusion. Murine bone marrow subsets include Fr. A–C' (B220<sup>+</sup>,

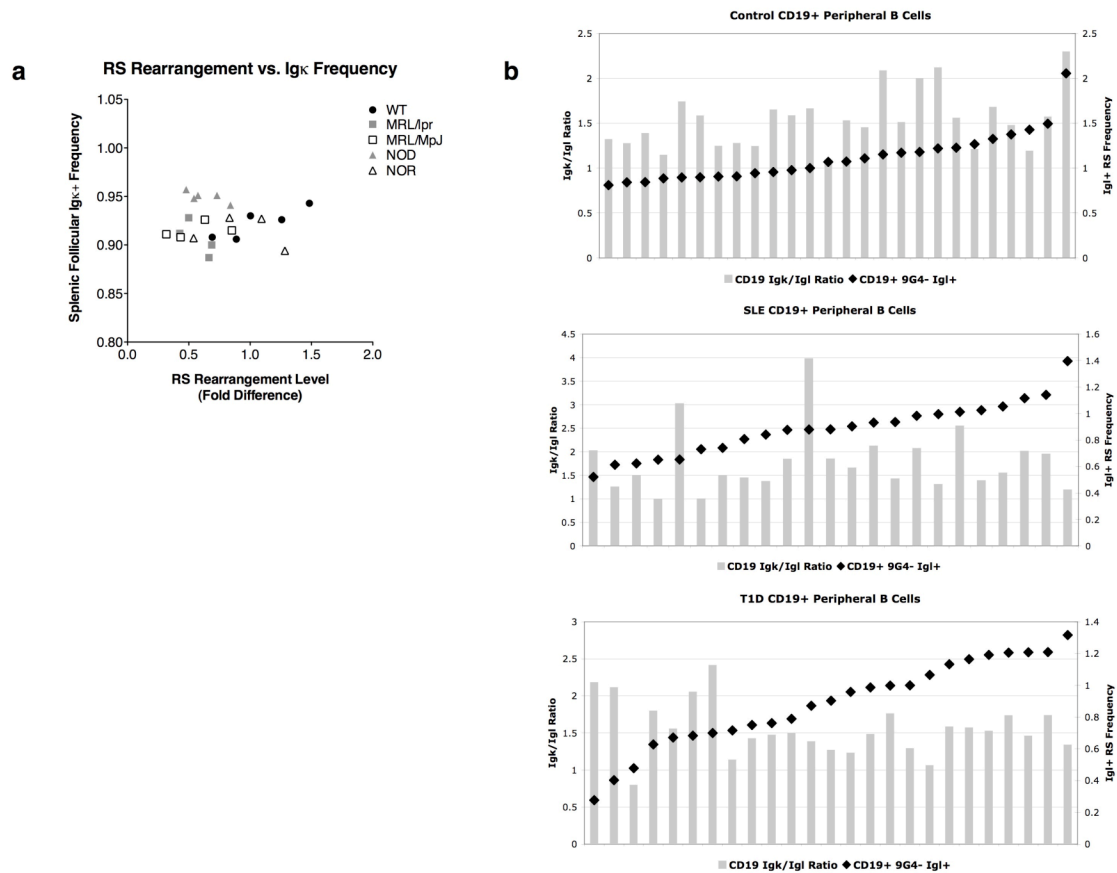
CD43<sup>+</sup>), Fr. D (B220<sup>+</sup>, CD43<sup>-</sup>, AA4.1<sup>+</sup>, IgM<sup>-</sup>), Fr. E (B220<sup>+</sup>, CD43<sup>-</sup>, AA4.1<sup>+</sup>, IgM<sup>+</sup>), and Fr. F (B220<sup>+</sup>, CD43<sup>-</sup>, AA4.1<sup>-</sup>, IgM<sup>+</sup>). Murine splenic populations were identified as follows: follicular B cells (B220<sup>+</sup>, AA4.1<sup>-</sup>, IgM<sup>int</sup>, CD23<sup>+</sup>), and marginal zone B cells (B220<sup>+</sup>, AA4.1<sup>-</sup>, IgM<sup>bri</sup>, CD23<sup>-</sup>). Due to poor reactivity of AA4.1 in NOD and NOR mice (Langmuir et al. 1993; Quinn et al. 2006), bone marrow populations in these mice were defined as follows: Fr. D (B220<sup>+</sup>, CD43<sup>-</sup>, IgD<sup>-</sup>, IgM<sup>-</sup>), Fr. E (B220<sup>+</sup>, CD43<sup>-</sup>, IgD<sup>-</sup>, IgM<sup>+</sup>), and Fr. F (B220<sup>+</sup>, CD43<sup>-</sup>, IgD<sup>+</sup>, IgM<sup>+</sup>). Human peripheral B cells were isolated on the basis of CD19,  $\kappa$ , and  $\lambda$  expression. To maximize efficient use of patient samples for additional experiments not described here, CD19<sup>+</sup> B cells marked by the anti-idiotypic monoclonal antibody 9G4, which recognizes V<sub>H</sub>4-34 heavy chain rearrangements (Isenberg et al. 1993), were excluded from human CD19<sup>+</sup> B cell populations described herein. 9G4Id<sup>+</sup> cells comprised 5.1%, 5.7%, and 4.2% of total CD19<sup>+</sup> B cells from control, SLE, and T1D subjects, respectively.

**Figure 2-3**



**Figure 2-3: Real-time PCR to monitor iRS-KDE rearrangements in B cell populations.** iRS-KDE rearrangements were amplified with 5'-ATT GAT GCT GCC GTA GCC-3' and 5'-AGG CTT CCT AGG GAG GTC AG-3' primers and detected with 5'-TCT GCA GCT GCA TTT TTG CCA-3' FAM-labeled hydrolysis probe. For each sample, the intronic region of the reference control gene  $\beta$ -actin was amplified in a separate well with forward primer 5'-CCC AGC ACA ATG AAG ATC AA-3' and reverse primer 5'-AGT ACT TGC GCT CAG GAG GA-3' and detected with a Cy5-labeled hydrolysis probe 5'-TGC CTG AGC TGA CCT GGG CA-3'. A cloned iRS-KDE rearrangement was serially diluted in fibroblast DNA to 0.7% RS<sup>+</sup> cells per 100ng of input DNA.

**Figure 2-4**

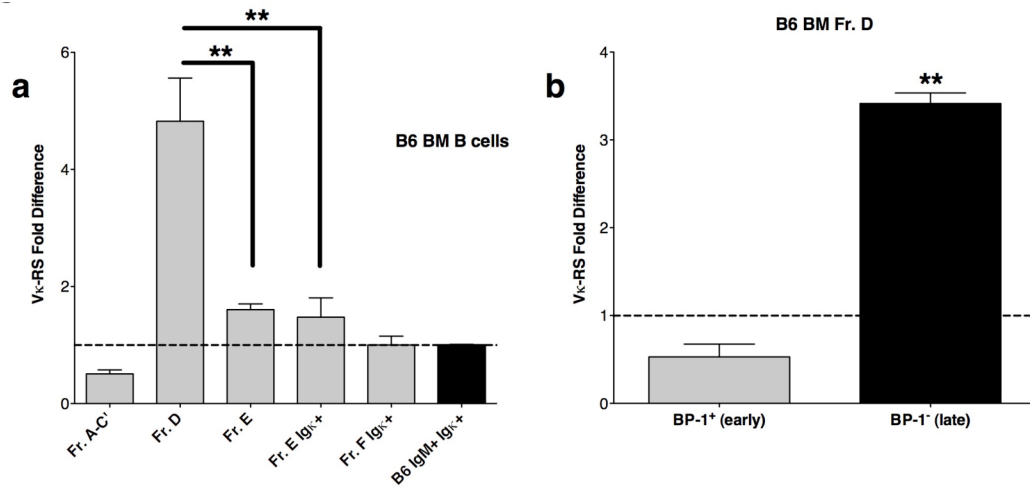


**Figure 2-4: RS rearrangement levels amongst  $\kappa^+$  B cells are independent of Ig $\kappa$ /Ig $\lambda$  ratios.** (a)  $V_{\kappa}$ -RS rearrangement levels in splenic Ig $\kappa^+$  follicular B cells from individual C57Bl/6 (black circles,  $n=5$ ,  $r=0.8$ ,  $p=0.1$ ), MRL/lpr (white squares,  $n=4$ ,  $r=-0.6$ ,  $p=0.4$ ), and NOD (gray triangles,  $n=5$ ,  $r=-0.7$ ,  $p=0.2$ ) mice compared to frequency of Ig $\kappa^+$  splenic follicular B cells. RS rearrangement levels are calculated as fold difference relative to the average RS level in C57Bl/6 splenic B220<sup>+</sup> IgM<sup>+</sup> Ig $\kappa^+$  B cells. (b) iRS rearrangement levels in CD19<sup>+</sup> 9G4<sup>-</sup> Igλ<sup>+</sup> (black diamonds, right y-axis) compared to CD19<sup>+</sup> 9G4<sup>-</sup> Igκ/Igλ ratios (gray bars, left y-axis). iRS rearrangement levels are depicted



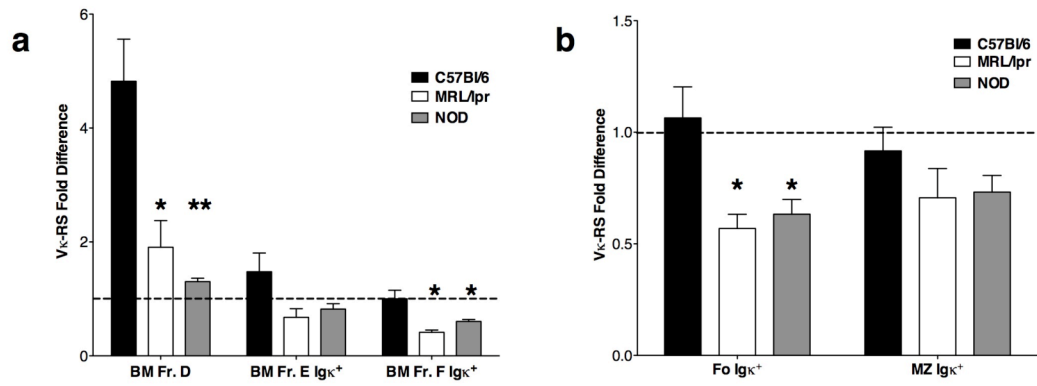
from healthy control subjects (top panel, n=26), SLE patients (middle panel, n=23), and T1D patients (bottom panel, n=25) and are calculated as RS frequency per genome copy.

**Figure 2-5**



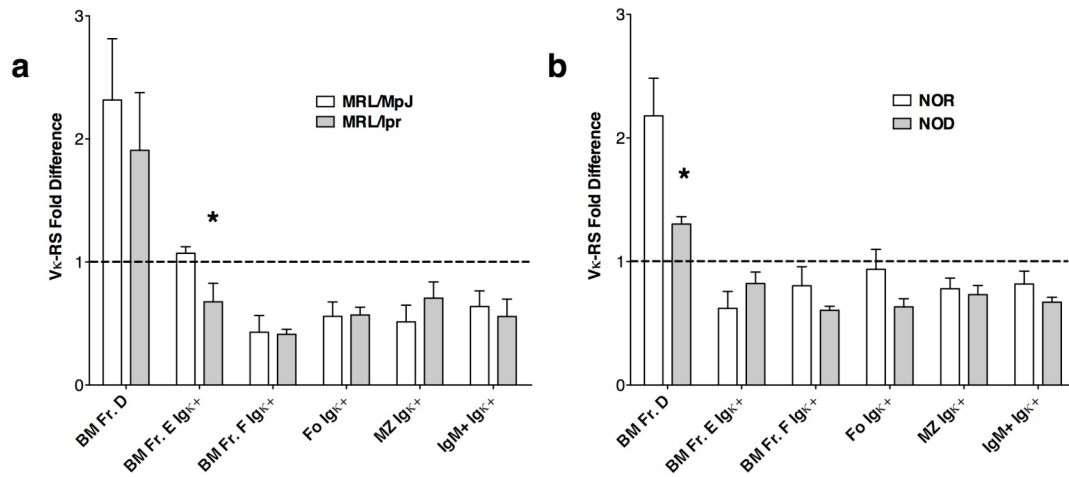
**Figure 2-5: RS rearrangement levels are highest in late pre-B cells.** (a) V $\kappa$ -RS rearrangement levels quantified in bone marrow (BM) subsets from C57Bl/6 mice (n=5). (b) V $\kappa$ -RS rearrangement levels assayed in less mature (BP-1 $^{+}$ , gray bar) and more mature (BP-1 $^{-}$ , black bar) Fr. D B cells of C57Bl/6 mice (n=3). All PCR reactions were performed in duplicate. Data are depicted as fold difference (+s.e.m., \*\* =  $p \leq 0.01$ ) relative to the average RS level in C57Bl/6 splenic B220 $^{+}$  IgM $^{+}$   $\kappa^{+}$  B cells (dashed line). Bone marrow B cell fractions were obtained by flow cytometry, as shown in Fig. 2-2.

**Figure 2-6**



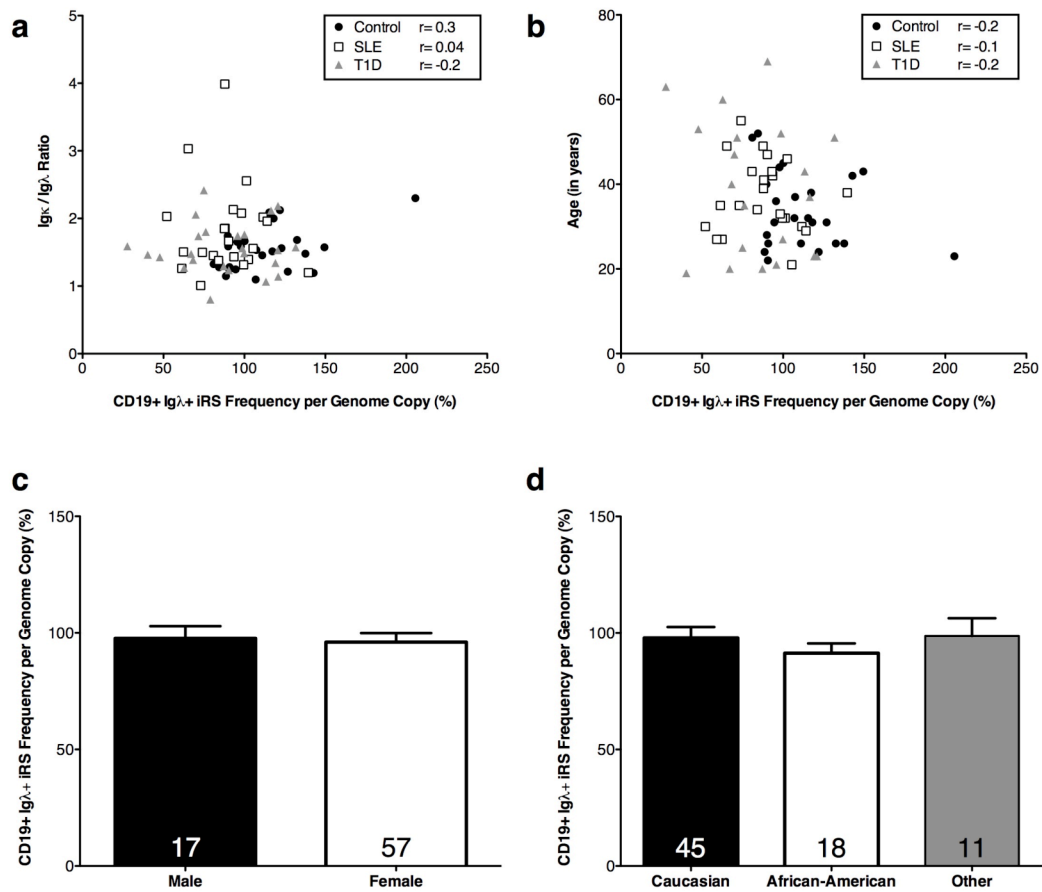
**Figure 2-6: Autoimmune prone mouse strains display reduced levels of RS rearrangement.** (a) V $\kappa$ -RS rearrangement levels in bone marrow subsets of MRL/lpr mice (white bars, n=4) and NOD mice (gray bars, n=5). Data from C57Bl/6 mice are included for comparison. (b) V $\kappa$ -RS rearrangement levels in  $\kappa^+$  splenic B cell subsets from C57Bl/6 (black bars, n=5), MRL/lpr (white bars, n=4), and NOD (gray bars, n=5) mice. All PCR reactions were performed in duplicate. Data are represented as fold difference (+s.e.m., \* = p<0.05 and \*\* = p<0.01) relative to the average RS level in C57Bl/6 splenic B220<sup>+</sup> IgM<sup>+</sup>  $\kappa^+$  B cells (dashed line).

**Figure 2-7**



**Figure 2-7: RS rearrangement levels in MRL/MpJ and NOR strains.** (a) Vκ-RS rearrangement levels in B cell fractions of the MRL/lpr control strain, MRL/MpJ (n=4, white bars) compared to MRL/lpr (n=4, gray bars). (b) Vκ-RS levels in B cell fractions of NOR mice (n=4, white bars) compared to NOD mice (n=5, gray bars). MRL/lpr and NOD data are the same data that are shown in Fig. 2-6. Data are represented as fold difference (+s.e.m., \* = p<0.05) relative to the average RS level in C57Bl/6 splenic B220<sup>+</sup> IgM<sup>+</sup> Igκ<sup>+</sup> B cells (dashed line). BM (bone marrow) and splenic B cell fractions are defined in Fig. 2-2.

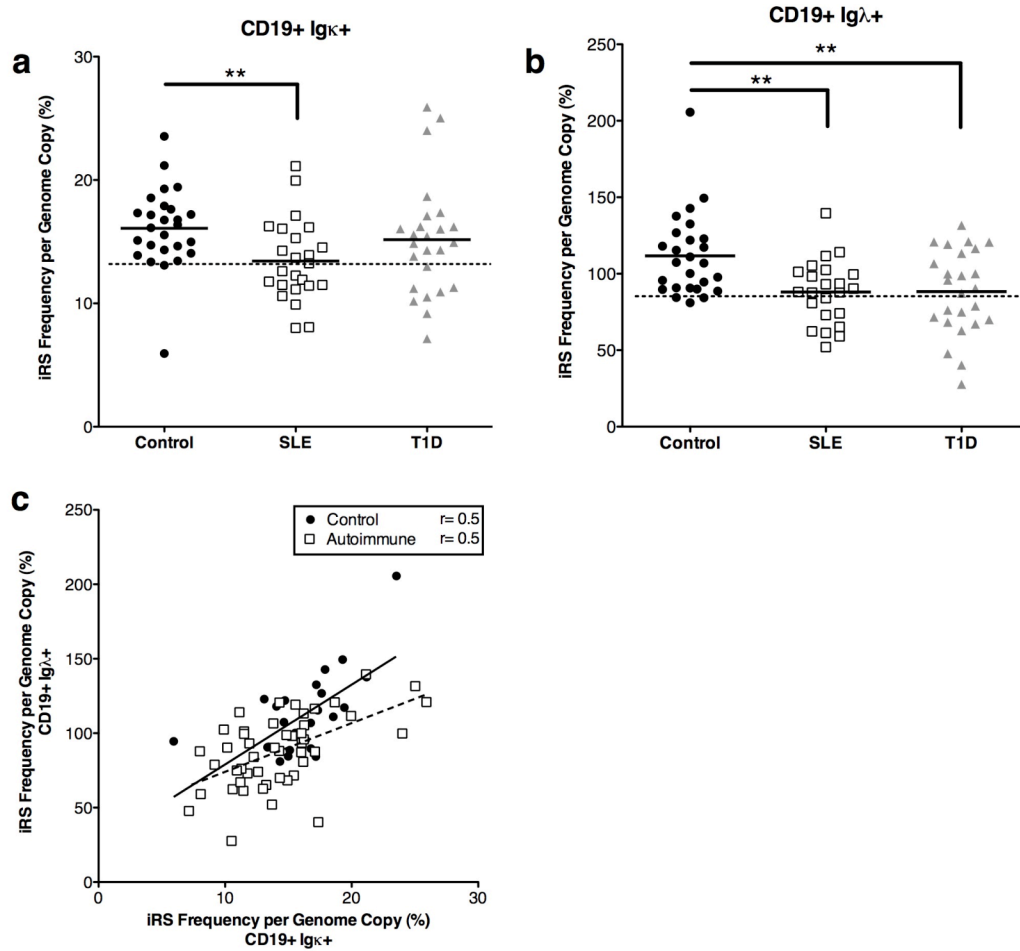
**Figure 2–8**



**Figure 2–8: RS rearrangement levels do not correlate with patient group demographics.** (a) iRS rearrangement levels in CD19<sup>+</sup> 9G4<sup>−</sup> Igλ<sup>+</sup> peripheral B cells from healthy controls (black circles, n=26), SLE patients (white squares, n=23), and T1D patients (gray triangles, n=25) compared to CD19<sup>+</sup> Igκ/Igλ ratios. (b) iRS rearrangement levels in CD19<sup>+</sup> 9G4<sup>−</sup> Igλ<sup>+</sup> peripheral B cells from healthy controls (black circles, n=26), SLE patients (white squares, n=23), and T1D patients (gray triangles, n=25) compared to subject age in years. (c) Mean iRS rearrangement frequencies compared between male (n=17) and female (n=57) subjects (+s.e.m., p=0.84). (d) Mean iRS rearrangement frequencies among Caucasian (n=45), African-American (n=18), and other (n=11,

includes Asian, Hispanic, and Native American) ethnic groups (+s.e.m.,  $p=0.67$ ). Data are depicted as iRS rearrangement frequency per genome copy (r-values represent Spearman correlation coefficients). See Table 2–1 for a detailed demographic summary.

**Figure 2–9**



**Figure 2–9: RS rearrangement levels are lower amongst SLE and T1D patients.** (a, b) iRS rearrangement frequencies as quantified in peripheral CD19<sup>+</sup> 9G4<sup>−</sup> κ<sup>+</sup> and CD19<sup>+</sup> 9G4<sup>−</sup> λ<sup>+</sup> B cells, respectively. Data represent the iRS levels in healthy control subjects (black circles, n=26), SLE patients (white squares, n=24 for κ<sup>+</sup>, n=23 for λ<sup>+</sup>), and T1D patients (gray triangles, n=25). Mean values are depicted as horizontal lines. The 10<sup>th</sup> percentile of iRS frequencies among control subjects is depicted as a dashed line. (c) iRS rearrangement frequencies in CD19<sup>+</sup> 9G4<sup>−</sup> κ<sup>+</sup> peripheral B cells compared to frequencies in CD19<sup>+</sup> 9G4<sup>−</sup> λ<sup>+</sup> cell from healthy controls (black circles, n=26) and SLE

and T1D patients (white squares, n=48). All PCR reactions were performed in duplicate. Data are depicted as iRS rearrangement frequency per genome copy. Trend lines are depicted for control (solid line, slope=5.4) and autoimmune patients (dashed line, slope=3.3; r-values indicate Spearman correlation coefficients, \*\* =  $p < 0.01$ ).



**Table 2-1**

	Control	SLE	T1D
Number of Subjects	26	24	25
Average Age (years)	33.8 (22-52)	37.3 (21-55)	38.5 (19-69)
% Female <sup>a</sup>	0.88	0.88	0.56
% Caucasian <sup>b</sup>	0.58	0.29	0.92
% African-American	0.27	0.42	0.04
% Other	0.15	0.29	0.04
Average $\kappa/\lambda$ Ratio (CD19 <sup>+</sup> )	1.5 (1.1-2.3)	1.8 (1.0-4.0)	1.6 (0.8-2.4)
Average B Cell Fraction (CD19 <sup>+</sup> )	0.11 (0.05-0.19)	0.10 (0.01-0.2)	0.10 (0.02-0.19)
Average Absolute B Cell Count (per $\mu$ l) <sup>c</sup>	221 $\pm$ 126	176 $\pm$ 163	190 $\pm$ 107

**Table 2-1: Demographic comparison among subject groups.** <sup>a, b</sup>Significant differences were found with respect to gender and race between the T1D group and control group ( $p < 0.05$  and  $p < 0.01$ , respectively) as well as between the T1D and SLE groups ( $p < 0.05$  and  $p < 0.001$ , respectively). <sup>c</sup>Absolute B cell counts were obtained by multiplying the white blood cell count (from the CBC) by the percentage lymphocytes (from the electronic differential) and by the CD19<sup>+</sup> fraction and are listed as averages  $\pm$  s.d. Absolute B cell counts represent the average of 25 control subjects, 23 SLE patients, and 24 T1D patients, respectively.

## **CHAPTER 3: Investigation of the Forces That Regulate Receptor Editing**

The text, figures, and experiments in this chapter were the work of Anil Panigrahi with the following exceptions. The reciprocal product isolation and characterization presented in Table 2 were performed by Hui Li of the Weigert Laboratory. The measurement of Ig $\lambda^+$  B cells in Lyn $^{-/-}$  mice presented in Figure 3–5b was performed by Andrew Gross of the DeFranco Laboratory.

## INTRODUCTION

Receptor editing is the process of ongoing antibody gene rearrangement which results in the generation of new antibody specificities. As seen in chapter 2, this critical tolerance mechanism may be aberrant in some autoimmune disease states, presumably permitting the development of autoreactive B cells that are capable of participating in autoimmune responses. However, the precise mechanism responsible for initiating receptor editing remains to be clearly defined.

Experiments by Hertz and Nemazee demonstrated that B cell receptor (BCR) ligation of bone marrow B cells *in vitro* results in upregulation of *Rag* mRNA, increased IgL rearrangement, and increased Ig $\lambda$  expression (Hertz and Nemazee 1997). The authors concluded that receptor editing is actively promoted by BCR signaling triggered by autoantigens. Further experiments by Retter and Nemazee supported this model. In these experiments, they exploited B cells in which the rearrangement history was retained on the chromosome. B cells that have inactivated C $\kappa$  by RS deletion, a common event in  $\lambda$  B cells (Durdik et al. 1984; Siminovitch et al. 1985), can harbor a V $\kappa$ -J $\kappa$  rearrangement on the C $\kappa$ -deleted allele. These V $\kappa$ -J $\kappa$  remnants were examined to determine if they contained in-frame (IF) rearrangements. They observed an elevated frequency of IF rearrangement remnants and thus proposed that receptor editing is an active, self-antigen driven process (Retter and Nemazee 1998). However, in this study they were unable to demonstrate that IF editing precursors were autoreactive.

Alternatively, Behrens and colleagues contend that recognition of self-antigens by immature B cells may not directly regulate receptor editing, instead proposing that absence of basal surface immunoglobulin (Ig) signaling reinitiates a transcriptional program which results in editing rearrangements (Tze et al. 2005). Indeed, loss of basal Ig signaling by Cre-mediated IgH deletion or by pharmacological blockade of tyrosine kinase signaling pathways both resulted in the upregulation of transcription factors

associated with earlier stages of B cell development, increased Rag transcription, and generation of new IgL rearrangements. Additionally, the Behrens group reported that self-antigen was capable of downregulating surface BCR and initiating the “de-differentiation” process (Schram et al. 2008). However, it is also possible that insufficient basal BCR signaling of immature B cells could lead to secondary IgL rearrangement. Thus, although these studies have improved our understanding of its regulation, a conclusive link between B cell autoreactivity and stimulation of receptor editing has yet to be demonstrated.

To address this issue we analyzed remnants of IgL rearrangements that have been edited, but are still retained in the chromosome. By examining these editing precursors in IgH transgenic mouse models where IgL specificities have been extensively characterized, we are able to determine if autoreactive light chains are edited more often. Additionally, the effect of aberrant BCR signaling on receptor editing was studied to discern whether positive BCR signals are required for efficient editing. Furthermore, the influence of genetic polymorphisms on editing was assessed in the hope of identifying key factors responsible for decreased RS rearrangement frequencies observed in the context of autoimmunity.

## **RESULTS**

### **RS rearrangement levels differ between autoreactive and non-autoreactive B cells**

It is unclear how or if receptor editing is linked to autoreactivity. Editing is driven either by the potential of the antibody heavy light chain pair to form autoantibodies (active model), or it occurs stochastically without regard for receptor specificity (passive model). To distinguish between active and passive editing, the RS rearrangement assay for estimating receptor editing frequencies (described in Chapter 2) was applied to the 56R mouse model, where the B cell repertoire has been

characterized extensively (Li et al. 2001; Sekiguchi et al. 2006; Liu et al. 2007). The 56R mouse carries a site-directed transgene (sd-tg) encoding an anti-DNA specific IgH. Using B6.56R<sup>+/-</sup> mice, one can distinguish B cells with an autoreactive IgH (IgM<sup>a+</sup>, mostly 56R expressing) and endogenous (IgM<sup>b+</sup>, 56R<sup>-</sup>) B cells (**Fig. 3-1a**). 56R-expressing B cells have a restricted light chain repertoire consisting of only a handful of light chains, termed editors, because they modify or reduce DNA binding (Li et al. 2001; Sekiguchi et al. 2006; Liu et al. 2007). This restriction in light chain usage could arise if 56R B cells with non-editor light chains were counter-selected or if B cells with non-editor light chains were subjected to more receptor editing. The former alternative predicts equal levels of RS rearrangement in IgM<sup>a+</sup> and IgM<sup>b+</sup> cells, whereas the latter predicts higher levels of RS rearrangement in IgM<sup>a+</sup> B cells. Consistent with an active model of receptor editing, a two-fold increase in RS rearrangement levels was observed in IgM<sup>a+</sup> B cells (**Fig. 3-1b**).

### **Recovery of editing precursors from splenic B cells**

The observation that RS rearrangement levels are elevated among 56R<sup>+</sup> B cells strongly suggests that autoreactivity plays a role in stimulating receptor editing. However, from this experiment alone the autoreactive specificity of 56R<sup>+</sup> cells bearing RS rearrangements cannot be confirmed. Thus, in order to more precisely define the original specificity of edited cells, a genetic approach focused on light chain precursor rearrangements was used.

The analysis of a lymphocyte's rearrangement history can provide insights into mechanisms that regulate ongoing V(D)J recombination. Different models predict different frequencies of productive (in-frame, IF) rearrangement precursors (Table 3-1). If feedback inhibition of rearrangement by a functional heavy light chain pair (H/L-STOP, (Alt et al. 1984; Cohn and Langman 1990)) were immediate and irreversible in all B cells,

no rearrangement precursors would be IF. The existence of receptor editing argues against this model since alleles with functional rearrangements can undergo further rearrangement (Harada and Yamagishi 1991; McCormack et al. 1993; Retter and Nemazee 1998; Holman et al. 2003). Conversely, if cells underwent secondary rearrangement without regard for the specificity or functional status of the primary rearrangement (the “leaky” H/L-STOP model), one would expect the fraction of IF rearrangement precursors to be approximately 33% (Harada and Yamagishi 1991). Yet, the complete absence of an H/L-STOP signal is unlikely, given experiments that demonstrate inhibition of further rearrangement (allelic exclusion) by antibody transgenes (Ritchie et al. 1984). The high frequency of cells with  $\kappa^+/\kappa^0$  genotypes also favors an active H/L-STOP (Coleclough et al. 1981).

V $\kappa$  editing can occur by deletion, resulting in the production of an episome (**Fig. 3-2a**), or by inversion, resulting in a reciprocal product (RP, **Fig. 3-2b**). A third editing pathway is RS deletion (**Fig. 3-2c**). Previous studies have analyzed V-J joins present on episomes (**Fig. 3-2a**, (Harada and Yamagishi 1991; McCormack et al. 1993; Holman et al. 2003)), however, these products are not maintained in cells and cannot be linked to a defined IgH chain. Therefore, we focused on rearrangement precursors that were retained on the chromosome. Of the two chromosomally retained pathways (inversion and RS deletion), RS deletion is more amenable to screening large numbers of rearrangements because the deleted allele can be identified on a short stretch of the chromosome (**Fig. 3-2b**). The editing rearrangement of interest utilizes a cryptic recombination signal sequence (RSS) within the J $\kappa$ -C $\kappa$  intron (iRS) which recombines with the  $\kappa$  locus Recombination Segment (RS), located 25kb downstream of C $\kappa$  (Durdik et al. 1984; Siminovitch et al. 1985). The resulting rearrangement product lacks C $\kappa$  (thereby inactivating the locus), but retains the V $\kappa$ -J $\kappa$  rearrangement.

To recover  $V_{\kappa}$ - $J_{\kappa}$  rearrangements on RS deleted kappa alleles, we purified genomic DNA from spleen fragments of homozygous 3H9 ( $3H9^{+/+}$ ) and 3H9/56R ( $56R^{+/+}$ ) IgH sd-tg mice (Erikson et al. 1991; Chen et al. 1995). The presence of the sd-tg on both alleles may reduce the chances of successful IgH chain editing, which could confound interpretation of antibody specificity. Using a PCR strategy similar to what has been described previously (Dunda and Corcos 1997; Retter and Nemazee 1998), we set up two types of assays to recover  $V_{\kappa}$ - $J_{\kappa}$  rearrangements on RS deleted kappa alleles (**Fig. 3-2b**). The first assay used a degenerate  $V_{\kappa}$  primer, Vs, to recover  $V_{\kappa}$ - $J_{\kappa}$  rearrangements without regard for their specificity (Schlissel and Baltimore 1989). The second assay used editor-specific  $V_{\kappa}$  primers ( $V_{\kappa}12$  in 3H9 and  $V_{\kappa}38c$  in 56R) to address if editor rearrangements could themselves be edited. We reasoned that an editor-specific rearrangement assay was necessary to recover adequate numbers of informative rearrangements in a model such as 56R, because nearly all functional  $V_{\kappa}$  genes are non-editors.

As depicted in Figure 3-2b, Ig $\kappa$  locus inactivation by RS deletion can occur via two major pathways, direct  $V_{\kappa}$  to RS rearrangement or rearrangement of the  $J_{\kappa}$ - $C_{\kappa}$  intron recombination signal sequence, iRS, to RS. Only the latter pathway retains the primary  $V_{\kappa}$ - $J_{\kappa}$  rearrangement on the chromosome. Unfortunately, iRS-RS rearrangements are harder to recover than  $V_{\kappa}$ -RS rearrangements, in part because the iRS-RS PCR product is several hundred base pairs larger. The PCR also may have introduced a selection bias in favor of rearrangements to  $J_{\kappa}5$  because such  $V_{\kappa}$ - $J_{\kappa}$  rearrangements are the smallest in size and most readily amplified. Alternatively, it is possible that rearrangements to  $J_{\kappa}5$  (the last  $J_{\kappa}$  segment available for rearrangement in the  $J_{\kappa}$  cluster) are more common on RS deleted alleles.

In estimating the frequency of IF editing precursors, we only counted rearrangements that were clonally independent. Several steps were taken in order to

ensure clonal independence. First, DNA was isolated from several mice per genotype. Second, each spleen was cut into fragments and DNA was extracted separately from each fragment. Third, junctional diversity of the iRS-RS rearrangement was used to identify unique rearrangements in cases where the  $V_{\kappa}$ -J $\kappa$  junctions appeared similar. While CDR3 diversity at Ig $\kappa$  is limited, junctional diversity at RS is considerable and consists mostly of small deletions (**Fig. 3-3**).

### **Analysis of editing precursors**

By comparing the frequency of IF editing precursors among editor  $V_{\kappa}$  rearrangements to the IF frequency among non-editor rearrangements, we can determine if the specificity of the primary rearrangement influences whether or not editing occurs. If autoreactivity stimulates receptor editing, we would expect fewer IF rearrangements among editor  $V_{\kappa}$  rearrangements than among non-editor  $V_{\kappa}$  rearrangements. In 56R<sup>+/+</sup> mice, 3 of the 11 (27%)  $V_{\kappa}$ -J $\kappa$  rearrangements using the editor  $V_{\kappa}38c$  were in-frame (**Fig. 3-4a**). This frequency is similar to the 33% predicted by a random editing process and is higher than expected if receptor editing is stimulated by autoreactivity. Indeed, when non-editor  $V_{\kappa}12$  rearrangements were examined in 56R<sup>+/+</sup> mice, 8 out of 32 (25%) rearrangements were IF (**Fig 3-4c**). The similarity in IF frequencies between editor and non-editor rearrangements in 56R suggests that either editing occurs without respect to BCR specificity or that the difference in specificities between editors and non-editors is not significant in the context of 56R. As the 56R heavy chain is highly specific for DNA, it is plausible that even 56R antibodies utilizing editor light chains still retain autoreactivity (see discussion).

When  $V_{\kappa}12$  rearrangements are studied in 3H9<sup>+/+</sup> mice, where they act as editors, 4 of 22 (18%) editing precursors were found to be in-frame (**Fig 3-4b**). This



frequency is nearly half of the 33% predicted if editing occurred randomly and suggests that these rearrangements are disfavored as substrates for receptor editing. Consistent with this interpretation is the fact that the 3H9 heavy chain confers a weaker anti-DNA reactivity than the 56R heavy chain implying that editor light chains may be more successful in abrogating autoreactivity when paired with 3H9 and thus accounting for the difference in IF frequencies between editors in 56R and 3H9 mice. The low frequency of IF editor rearrangement precursors is not due to inefficient recombination of editor V $\kappa$ s because the IF rearrangement frequency of the same V $\kappa$  seems to depend upon the IgH chain context: 18% of V $\kappa$ 12 (editor) rearrangements in 3H9 are IF compared to 25% of V $\kappa$ 12 rearrangements (non-editor) in 56R. However, this difference is not statistically significant due to the small sample size ( $p=0.4$ ); demonstrating significance given the current frequencies would require characterization of over 200 precursors. Nevertheless, the result indicates a trend toward a decreased IF frequency among non-autoreactive editing precursors.

A caveat to these data is that despite having the sd-tg on both IgH chain alleles, the transgene could still be edited (Yunk et al. 2009). To exclude this possibility, 56R<sup>+</sup> hybridomas were analyzed for the presence of functional V $\kappa$ -J $\kappa$  joins on RS deleted Ig $\kappa$  alleles. Five out of ten (50%) RS deleted V $\kappa$ -J $\kappa$  rearrangements were IF and all rearrangements were to non-editor V $\kappa$ s (**Fig. 3-4d**). When the hybridoma and spleen data are combined, 31% (13/42) of non-editor rearrangements were found to be IF.

### **Identifying editing precursors among reciprocal products**

One potential limitation to the data obtained from spleen DNA and hybridoma panels derived from 3H9 and 56R mice is that V $\kappa$ -J $\kappa$  rearrangements on the second kappa allele could influence editing on the first. Therefore we characterized hybridoma reciprocal products isolated from mice hemizygous for J $\kappa$ -C $\kappa$  deletion (J $\kappa$ <sup>-/+</sup>; (Chen et

al. 1993)). These mice also possess heavy chain site-directed transgenes (sd-tgs) 3H9/GL, 3H9 or 56R (Erikson et al. 1991; Chen et al. 1995; Li et al. 2003).

For a hybridoma to have an informative reciprocal product (RP), it must satisfy two criteria. First, it must retain the heavy chain transgene ( $tg^+$ ). For 3H9/GL- $\kappa^{+/-}$ , 3H9, and 56R this is usually the case (83–95% of hybridomas are  $tg^+$ ) (Erikson et al. 1991; Chen et al. 1995; Li et al. 2003). Second, the orientation of the Ig $\kappa$  chain editor must be such that the RP is retained on the chromosome. For example, when the editor, V $\kappa$ 12, replaces a V $\kappa$ -J $\kappa$ 1 rearrangement by inverting to J $\kappa$ 2, we can characterize the V $\kappa$ -J $\kappa$ 1 RP because it is retained on the chromosome (**Fig. 3–2c**). The germline orientations of all V $\kappa$ s are known (Thiebe et al. 1999), so the post-rearrangement status of a V $\kappa$  can be inferred. We chose to focus mainly on J $\kappa$ 2 editor rearrangements because rearrangements to downstream J $\kappa$  segments (J $\kappa$ 4 or J $\kappa$ 5) could arise through more complex rearrangement pathways. The study of J $\kappa$ 2 editors was facilitated by having a large collection of hybridomas that were edited by rearrangement to J $\kappa$ 2.

To determine the fraction of IF RP, partial sequences of RP to J $\kappa$ 1 were obtained by PCR with Vs and J $\kappa$ 1inv primers (**Fig. 3–2c**). These sequences include the V $\kappa$ -J $\kappa$ 1 junction, allowing assignment of the reading frame. Our analysis of V $\kappa$ -J $\kappa$ 1 rearrangements in RP of heavy chain  $tg^+$  J $\kappa$  $^{+/-}$  hybridomas revealed that 5 out of 13 (38%) were IF (Table 3–2). If autoreactive cells had a higher death rate than cells with non-self rearrangements, one would predict fewer rather than more IF RPs (Table 3–1). Although our analysis revealed that RPs are rare among peripheral B cells, the abundance of functional rearrangements in RS deleted alleles and chromosomal RPs in this study is consistent with a high frequency of autoreactivity in the pre-edited repertoire and/or, paradoxically, that B cells harboring autoreactive light chains are significantly favored.

### **Lyn-mediated inhibition of BCR signaling does not influence receptor editing**

If receptor editing is actively stimulated by BCR signaling that results from autoantigen ligation, enhanced BCR signaling may lead to increased receptor editing by decreasing the required signaling threshold. Alternatively, enhanced signaling may lead to increased deletion of immature autoreactive B cells and consequently result in lower levels of editing among survivors (Cornall et al. 1998; Braun et al. 2000). To address this issue, RS rearrangement levels were measured in bone marrow and splenic B cells from Lyn<sup>-/-</sup> mice. Lyn is a Src family tyrosine kinase that not only phosphorylates ITAMs of BCR components, but also engages immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on B cell co-receptors which ultimately leads to a reduction in BCR signaling.

The absence of Lyn activity did not affect RS recombination in pre-B cells (Fr. D), as RS rearrangement levels were similar to those in wild-type mice (**Fig 3-5a**). Additionally, loss of Lyn inhibition did not alter the decrease in RS levels seen at the immature B cell stage (Fr. E) indicating that the loss of RS<sup>+</sup> cells at this transition is independent of Lyn. Similarly, RS rearrangement levels among splenic transitional B cells were unaffected (**Fig 3-5a**). Taken together these data suggest that Lyn is not required for normal receptor editing and does not substantially influence tolerance checkpoints during B cell development. Consistent with this interpretation, frequencies of Igλ<sup>+</sup> B cells decreased normally through B cell development in Lyn<sup>-/-</sup> mice, demonstrating that these cells were appropriately counter-selected (**Fig 3-5b**).

### **Autoimmune associated polymorphism in PTPN22 does not affect receptor editing**

Several studies have discovered that a single nucleotide polymorphism (SNP 1858C to T) in the gene encoding the protein tyrosine phosphatase N22 (PTPN22, also known as lymphoid tyrosine phosphatase, Lyp) is correlated with multiple autoimmune diseases in humans, including type-1 diabetes (T1D) and systemic lupus erythematosus

(SLE) (Bottini et al. 2004; Kyogoku et al. 2004). The 1858C/T SNP causes a conformational change in the interaction site of PTPN22 with the protein tyrosine kinase CSK and results in increased inhibition of TCR signaling (Cloutier and Veillette 1999; Vang et al. 2005). PTPN22 is also expressed in B cells, however, its role in B cell signaling and activation is still being determined. Interestingly, the PTPN22 1858T allele is prevalent in two patient groups in which we observed reduced receptor editing levels (SLE and T1D, see Chapter 2). Furthermore, Buckner and colleagues recently reported that the PTPN22 SNP resulted in decreased numbers of memory B cells which additionally displayed decreased responses to BCR stimulation (Rieck et al. 2007). Thus, although a role for PTPN22 in B cell development has not been established, it may be possible that increased inhibition of BCR signaling in immature B cells may hinder receptor editing by disrupting autoantigen mediated stimulation.

To investigate the role of the PTPN22 1858T variant on receptor editing, healthy individuals were separated based upon their SNP status and the degree of RS rearrangement in peripheral B cells of each group was assessed. Unlike the previous experiments, none of the individuals tested had signs of autoimmunity. Therefore if the presence of the SNP correlated with decreased RS rearrangement, it would additionally suggest that the RS assay may be useful as a predictor of autoimmune disease development. However, no difference in RS rearrangement frequencies between individuals carrying the disease-associated 1858T allele and those with the more common 1858C allele were observed in mature peripheral B cells (CD19<sup>+</sup> CD24<sup>low</sup> CD38<sup>int</sup>, **Fig. 3-6a**). Moreover, RS rearrangement levels in both Igκ<sup>+</sup> and Igλ<sup>+</sup> B cells were similar to levels from the healthy individuals described in Chapter 2. Despite this finding, it is possible that defects in receptor editing may not be detectable among mature B cells, as peripheral tolerance mechanisms in these healthy individuals may eliminate minimally edited B cells from the circulation. To address this issue, RS

rearrangement levels of immature B cells (CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup>) from the same individuals were assayed. Similar to mature B cells, RS levels were found to be equivalent between the two SNP groups (Fig. 3-6a) indicating that the 1858T variant does not affect rearrangement levels among immature peripheral B cells. Additionally, no consistent intra-individual changes between RS frequencies in immature and mature B cells were detected (Fig. 3-6b), suggesting that if increased PTPN22 activity leads to the development of minimally edited B cells, such cells are likely deleted in the bone marrow or prevented from entering the peripheral circulation.

### **NOD *Idd* loci 3 and 5 do not contribute to decreased RS rearrangement levels**

As discussed in Chapter 2, NOD mice, a model of type-1 diabetes, demonstrate decreased levels of RS rearrangement suggesting that defects in receptor editing may contribute to their disease susceptibility. The cause of the decrease in editing is likely complex, as extensive investigation into the genetic basis of diabetes in these mice has revealed over 20 *Idd* (insulin-dependent diabetes) loci that contribute to disease (Ridgway 2006). However, in addition to insulinitis, NOD mice also experience exocrine gland dysfunction resulting from autoimmune damage of salivary and lacrimal glands. Moreover, this autoimmune exocrinopathy (AEC, also known as Sjögren's syndrome) is correlated with *Idd5* and *Idd3*, located on chromosomes 1 and 3, respectively (Brayer et al. 2000). Importantly, when these loci were bred onto C57Bl/6 mice, the resulting AEC mice display a Sjögren's syndrome-like phenotype, with lymphocytic infiltration of exocrine glands, decreased secretory function, and serum autoantibody production (Cha et al. 2002). Given the shared B cell involvement in both disease processes and the common genetic loci between NOD and AEC mice, we hypothesized that receptor editing may also be altered in AEC mice and if so, could help restrict the search for novel elements that regulate receptor editing.

Therefore RS rearrangement levels were measured in 3-month old AEC mice for comparison with NOD mice to determine if impaired receptor editing is also correlated with *Idd3* and 5 (**Fig. 3-7**). In contrast to NOD mice that display decreased RS rearrangement levels in the majority of B cell subsets tested, AEC mice displayed RS levels similar to wild-type C57Bl/6 mice in all subsets. This result suggests that genes located in *Idd3* and *Idd5* do not directly influence receptor editing levels or at the least require additional defects in order to affect RS rearrangement.

## DISCUSSION

Receptor editing is a crucial tolerance mechanism of developing B cells, however, the precise regulation of this process is still unclear. Mounting evidence suggests that editing occurs in response to autoreactivity of immature B cells, however, it is also possible that increased residence time of B cells in the bone marrow may lead to prolonged Rag activity and secondary IgL rearrangement. If autoreactivity drives receptor editing, then B cells known to express anti-self BCRs should harbor signs of more extensive IgL rearrangement. Indeed, an increased RS rearrangement frequency was observed in B cells with autoreactive heavy chains (56R<sup>+</sup> B cells) relative to those with wild-type heavy chains. Because RS rearrangements do not encode a protein that can be selected, this result indicates that the presence of an autoreactive heavy chain increases the likelihood of ongoing light chain rearrangement and is consistent with other reports favoring an active, autoantigen driven model of receptor editing (Hertz and Nemazee 1997; Schram et al. 2008). However, additional experiments are required to rule out developmental changes caused by expression of a preformed IgH which could indirectly lead to increased RS rearrangement (see Chapter 4).

Additional IgL rearrangement can also occur if V<sub>κ</sub>-J<sub>κ</sub> recombination results in a non-functional light chain (out-of-frame rearrangement). However, since feedback

inhibition (H/L STOP) is known to be incomplete, rearrangement can continue despite the presence of functional, in-frame IgL rearrangements. An active model of editing would predict a bias among these rearrangements, as autoreactive light chains should be edited more often and thus be more prevalent among edited IF IgL rearrangements. To test this prediction, a detailed genotypic analysis of receptor editing precursors in the form of RS deleted and inverted (RPs)  $V_{\kappa}$ - $J_{\kappa}$  rearrangements was performed. B cells from three different heavy chain knock-in mouse models (3H9 GL, 3H9, and 56R) were studied. By fixing the heavy chain, it was possible to make inferences regarding the reactivity (autoreactive vs. non-autoreactive) of the edited light chain rearrangements. The analysis revealed a significant number of IF edited rearrangements and, among IF rearrangement precursors, an enrichment of autoreactive light chains.

In support of an autoreactivity-driven model, we observed a trend toward an increase in the IF frequency of  $V_{\kappa}12$  precursors when acting as non-editors (25%) compared to editors (18%). The difference in IF frequency between these two situations indicates that autoreactive specificities influence receptor editing and is incompatible with a model of random secondary rearrangement ("leaky" H/L STOP). In fact, if autoreactive rearrangements led to deletion of the B cell, then the predicted frequency of IF autoreactive precursors should be less than that of non-self precursors. That the IF frequency of non-editor  $V_{\kappa}s$  is higher supports a model in which cells with autoreactive  $V_{\kappa}s$  are favored. Additionally, in surveying editing precursors from heavy chain transgene expressing hybridomas, we found an even greater frequency of IF rearrangements (50%), all of which involved non-editor light chains. Among RS deleted  $\kappa$  alleles in wild type mice, Retter and Nemazee found that 47% were IF. The abundance of IF  $V_{\kappa}$ - $J_{\kappa}$  precursors in both of these studies is incompatible with the strict H/L-STOP model of light chain rearrangement (Table 3-1). It is also inconsistent with leaky H/L-STOP model, which would predict that no more than 33% of edited  $V_{\kappa}$ - $J_{\kappa}$  rearrangements

would be IF. Taken together these findings support a model where autoreactive rearrangements are selectively targeted for editing, and along with the high IF frequencies of non-editor precursors, lend further support for the finding that the fraction of the normal B cell repertoire that is potentially autoreactive is high (Wardemann et al. 2003).

Despite the findings in support of an autoreactivity-driven model of receptor editing, examination of editor precursor rearrangements in 56R<sup>+/+</sup> mice revealed significantly higher IF frequencies than expected. Out of 11 V $\kappa$ 38c rearrangements isolated, 3 (27%) were found to be IF. This frequency is 1.5 times greater than the IF frequency of editors in 3H9<sup>+/+</sup> mice and much closer to the 33% frequency predicted by a random model of receptor editing. There are several possibilities that can account for this IF frequency. First, as described previously, the method of recovering editing precursors after RS rearrangement can lead to biases toward smaller precursor rearrangements that often involve J $\kappa$ 5. This bias could influence the IF frequency if J $\kappa$  usage altered the specificity of the light chain as an editor. Indeed, previous studies have demonstrated that V $\kappa$ 38c editors in 56R tend to have a restricted J $\kappa$  usage (Li et al. 2001; Sekiguchi et al. 2006). This restriction, however, is generally to J $\kappa$ 4 or J $\kappa$ 5 and as all of the V $\kappa$ 38c precursors isolated from 56R<sup>+/+</sup> were rearranged to J $\kappa$ 5, it is likely that the IF precursors would have functioned appropriately as editors.

Alternatively, the additional arginine residue introduced in the 56R heavy chain may make editor light chains ineffective in fully nullifying the anti-DNA specificity. Thus, despite being expressed by mature peripheral B cells, editor light chains in 56R may still retain autoreactivity. Weigert and colleagues tested monoclonal antibodies (mAbs) from 56R hybridomas and found that despite incorporating editor light chains, several bound dsDNA (Li et al. 2001). In their hybridoma survey, they observed that 56R antibodies mainly used three different editor light chains: V $\kappa$ 20, V $\kappa$ 21D, and V $\kappa$ 38c.



Interestingly, binding studies revealed that although the majority of 56R antibodies associated with V $\kappa$ 20 or V $\kappa$ 21D did not bind dsDNA, 4 out of 7 hybridomas expressing the editor V $\kappa$ 38c still bound DNA. In these cases DNA reactivity was attributed to allelic inclusion at  $\lambda$ , as the hybridomas were positive for  $\lambda$ 1 rearrangements by PCR and ELISA. A mechanism for generating this allelic inclusion has been proposed by Khan *et al.* whereby V $\kappa$ 38c associated 56R heavy chains bind not only DNA, but also a Golgi related antigen (Khan *et al.* 2008). A consequence of this reactivity is sequestration of IgM molecules from the cell surface, which may allow for continued light chain rearrangement and allelic inclusion. Indeed, marginal zone B cells, a population thought to be enriched for allelically included B cells (Li *et al.* 2002), demonstrated increased levels of V $\kappa$ 38c rearrangement. The findings that autoreactivity and allelic inclusion are associated with V $\kappa$ 38c light chains in 56R mice provide a compelling explanation for the high IF frequencies observed among V $\kappa$ 38c editing precursors. Although peripheral B cells from 56R mice express V $\kappa$ 38c light chains, V $\kappa$ 38c does not appear to function as a true editor in the sense of abrogating autoreactivity. Consequently, the high IF frequency of V $\kappa$ 38c precursors is not inconsistent with an autoreactivity driven model for receptor editing and in fact may support it. Analysis of V $\kappa$ 20 or V $\kappa$ 21D precursor rearrangements in 56R may prove more informative as these light chains have been shown to reduce self-reactivity (Liu *et al.* 2008) and thus could provide more conclusive support for an autoreactivity-induced model of editing.

Overall, 33% of edited autoreactive rearrangements characterized in this study were in-frame. Moreover, when 56R-expressing hybridomas were surveyed without respect to V $\kappa$  usage, 50% of editing precursors were in-frame. This frequency is similar to that observed by Retter and Nemazee in their survey of wild-type  $\lambda$ -expressing hybridomas, where they found that 47% of edited rearrangements were in-frame. However, the Hogquist laboratory reported a lower frequency of in-frame

rearrangements (~26%) in excision circles from HY TCR $\beta$  transgenic mice (Holman et al. 2003). A key difference between these data sets is the types of rearrangements each represent. Unlike inverted or RS deleted rearrangements, excision circles are not maintained through replication cycles. Consequently, the joins on these episomes represent a full spectrum of rearrangements, including those in cells that will not pass negative selection. Thus the low IF frequency among episomal rearrangements suggests that the rearrangement process itself is not biased towards a higher IF frequency. Rather, B cells that successfully edit (pass selection) have a higher proportion of IF editing rearrangement precursors. This implies that B cells with productive rearrangements are favored. Yet this favoritism is likely not only due to having an IF primary rearrangement, since a potential decrease in IF frequencies of editor V $\kappa$ s (18%) suggests a bias based upon specificity. One possibility to account for this difference is the positive selection of autoreactive receptors before editing takes place. This idea is supported by recent studies from the Jumaa lab where expression of polyreactive BCRs lead to enhanced survival and expansion of immature B cells (Meixlsperger et al. 2007; Kohler et al. 2008).

Thus, it appears that selection acts upon autoreactive V $\kappa$ -J $\kappa$  rearrangements prior to tolerance induction and again after editing. But why would it be useful to favor the production of what is usually an autoreactive BCR only to modify or abrogate its auto-specificity in a subsequent rearrangement step? The generation of B cells that can recognize self may help optimize the immune repertoire to recognize altered self. This argument has long been used to rationalize positive selection in T cells (Jameson et al. 1995). Yet the molecular mechanism of achieving optimal recognition of altered self is still poorly understood. One potential function of the antibody light chain (or TCR $\alpha$ ) editing is to fine-tune the repertoire so that it is weakly anti-self. Self-specificity could be maintained by the dominant influence of the heavy chain, which is then modified to a

lesser degree by light chain editing. Alternatively, editing could result in light chain allelic inclusion, therefore promoting multi-reactivity that could be beneficial in detecting antigens common to various pathogens.

If receptor editing is indeed stimulated by autoantigen mediated BCR signaling, perturbation of the signaling pathway should influence the degree of receptor editing. To address this question, the effect of two signaling molecules on receptor editing was assessed. Lyn is a tyrosine kinase that normally functions to inhibit BCR signaling. However, Lyn<sup>-/-</sup> B cells did not show differences in receptor editing levels compared to wild-type B cells, indicating that Lyn-mediated inhibition is not required for effective regulation of receptor editing. Interestingly, Lyn<sup>-/-</sup> mice develop a lupus-like phenotype with increased serum IgM levels, serum autoantibodies, and glomerulonephritis (Hibbs et al. 1995). These defects appear to be the result failed peripheral tolerance mechanisms, as receptor editing and deletion of immature B cells are intact (Gross et al. 2009). Similarly, a polymorphism in PTPN22, which is found in high frequencies among humans with autoimmune disease, did not affect receptor editing as measured by RS rearrangement. This phosphatase serves to inhibit TCR signaling in T cells and may play a similar role in B cells, but the increased activity of 1858T variant did not affect RS rearrangement frequencies.

As both of the molecules serve to temper positive BCR signaling, these results suggest that either Lyn and/or PTPN22 do not function in immature B cells or that the regulation of receptor editing does not involve positive BCR signaling. If the latter alternative is true, it suggests that receptor editing is not directly stimulated by BCR signaling. However, this does not preclude receptor editing from occurring in response to autoantigen ligation. As proposed by Behrens and colleagues, BCR ligation in immature B cells may lead to loss of BCR surface expression and de-differentiation of immature B cells and induction of secondary IgL rearrangement. This back-

differentiation process appears to result from loss of basal signaling from surface BCR (Tze et al. 2005), suggesting that positive signaling is not required for receptor editing. Consistent with the results from the Lyn and PTPN22 studies, Schram *et al.* found that mimics of positive BCR signaling in immature B cells impaired receptor editing rather than promoting it (Schram et al. 2008).

In addition to investigating the nature of the stimulus for receptor editing, the studies presented here attempted to identify genetic defects that are responsible for decreased receptor editing observed in conditions of autoimmunity. To that end RS rearrangement levels in the AEC mouse strain, a model of Sjögren's syndrome, were characterized. AEC mice develop autoimmune disease but only have 2 of the over 20 *Idd* loci from the NOD strain correlated with development of diabetes. If AEC mice also demonstrated defects in RS rearrangement it could focus the search for additional factors that influence receptor editing. However, RS rearrangement levels in these mice were found to be equivalent to those in wild-type mice. AEC mice carry the NOD *Idd3* locus, located on chromosome 3, which has been implicated in lymphocyte dysfunction. Specifically, this locus has been attributed with defects in T cell suppression likely due to impaired IL-2 production (Yamanouchi et al. 2007). Additionally, the other NOD derived locus expressed in AEC mice, *Idd5* on chromosome 1, has been implicated in aberrant T cell activation. Mice carrying the NOD *Idd5* allele, demonstrate surface expression of an altered CTLA-4 isoform, an important suppressor of TCR signaling, which accelerates development of T1D (Luhder et al. 1998; Wicker et al. 2004). Thus it appears that the NOD-derived *Idd* loci present in AEC mice likely cause aberrant T cell activation which contributes to development of autoimmunity (Lundholm et al. 2006).

Despite the evidence for T cell effects, *Idd5* has also been reported to affect the diabetogenic activity of B cells in NOD mice. Previous studies have found that T1D in NOD mice develops in part due to B cell intrinsic effects (Serreze et al. 1996;

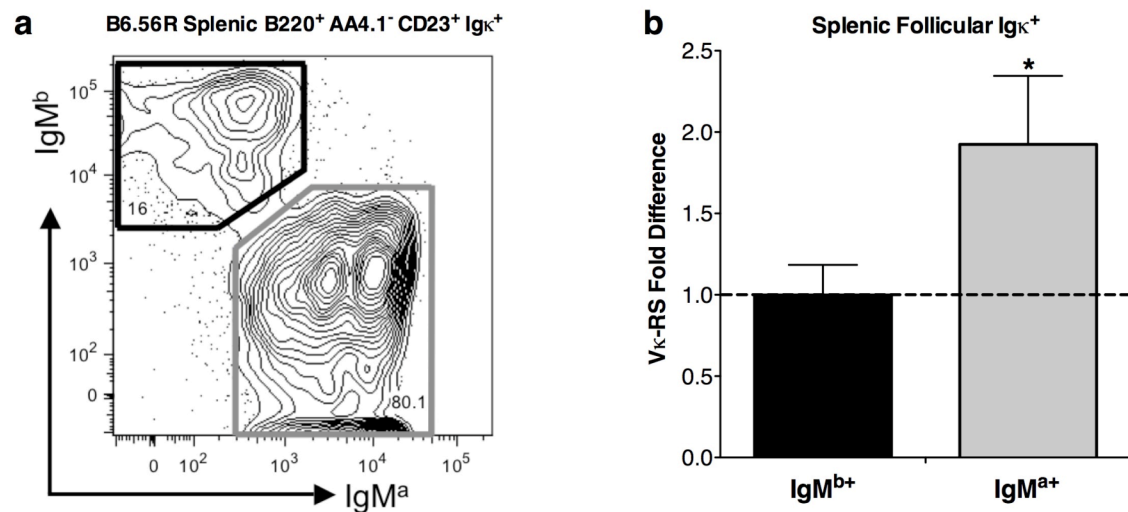
Noorchashm et al. 1997). NOD B cells were found demonstrate defects in clonal deletion of immature B cells and impaired anergy (Silveira et al. 2004). Interestingly, NOR mice, which display decreased RS rearrangement despite being diabetes resistant, were found to anergize autoreactive B cells normally. Moreover, this effect was found to correlate with differences in *Idd5* and *Idd9/11* (Silveira et al. 2006). As both NOD and NOR mice displayed decreased levels of RS recombination, this indicates that the B cell effect of NOD *Idd5* likely does not influence receptor editing and is consistent with the results from AEC mice. Taken together these results suggest that *Idd3*, 5, and 9/11 do not influence receptor editing.

The results described here are consistent with a model of receptor editing that is biased towards autoreactive BCRs. Analysis of BCR signaling mediators, however, suggests that positive BCR signals may not be required for effective receptor editing. Furthermore, several genetic polymorphisms correlated with autoimmune disease were found not to influence receptor editing, indicating that additional factors are responsible for impaired receptor editing observed in mouse models and subsets of patients with autoimmune disease.

## **ACKNOWLEDGEMENTS**

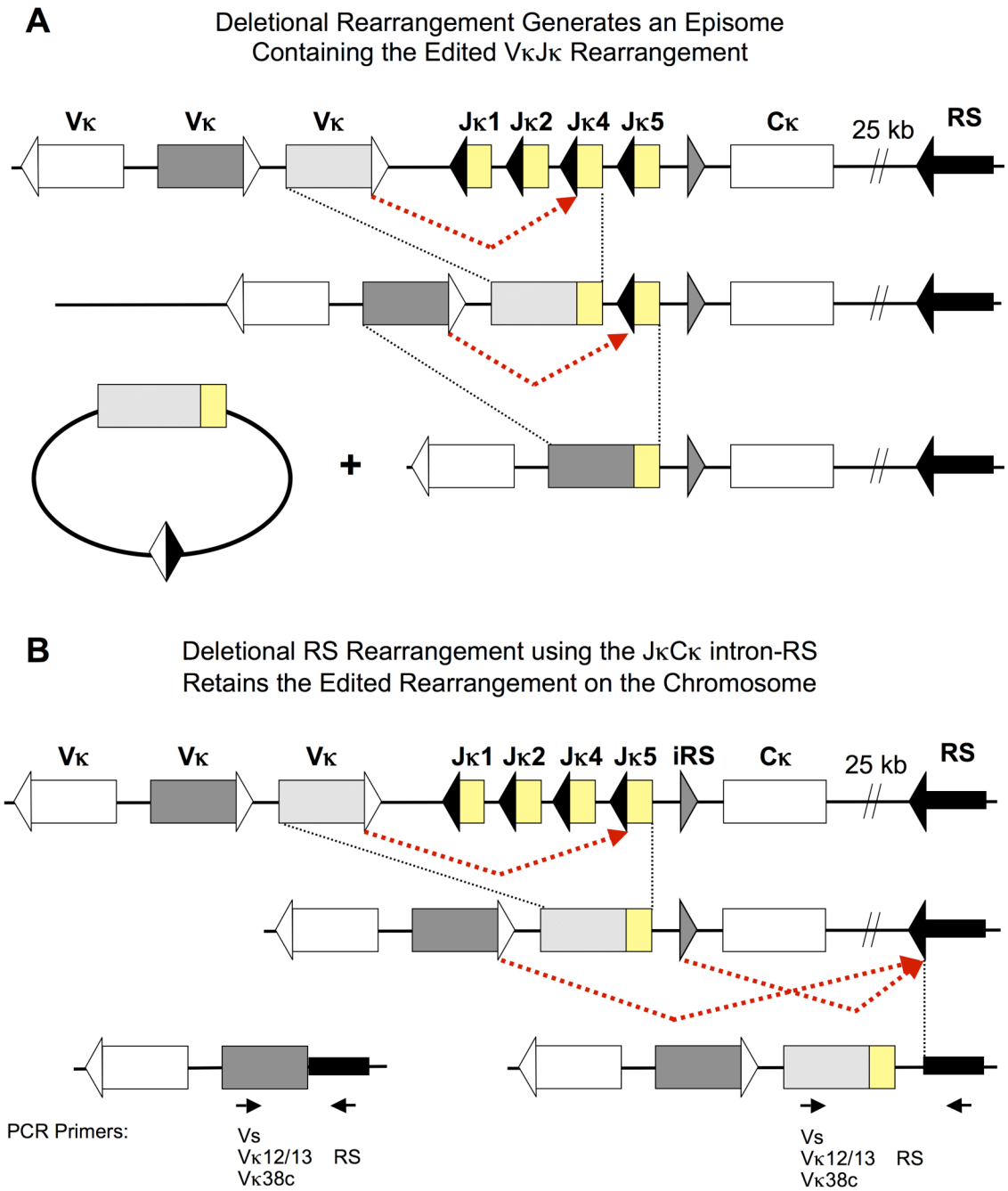
I would like to thank Almut Meyer-Bahlburg, Archana Brahmandam, and David Rawlings from the University of Washington, Seattle Children's Research Institute and Jane Buckner from the Benaroya Research Institute at Virginia Mason for assistance with patient recruitment and peripheral B cell isolation. Additionally, I would like to thank Wenzhao Meng for generously offering AEC mice for my experiments.

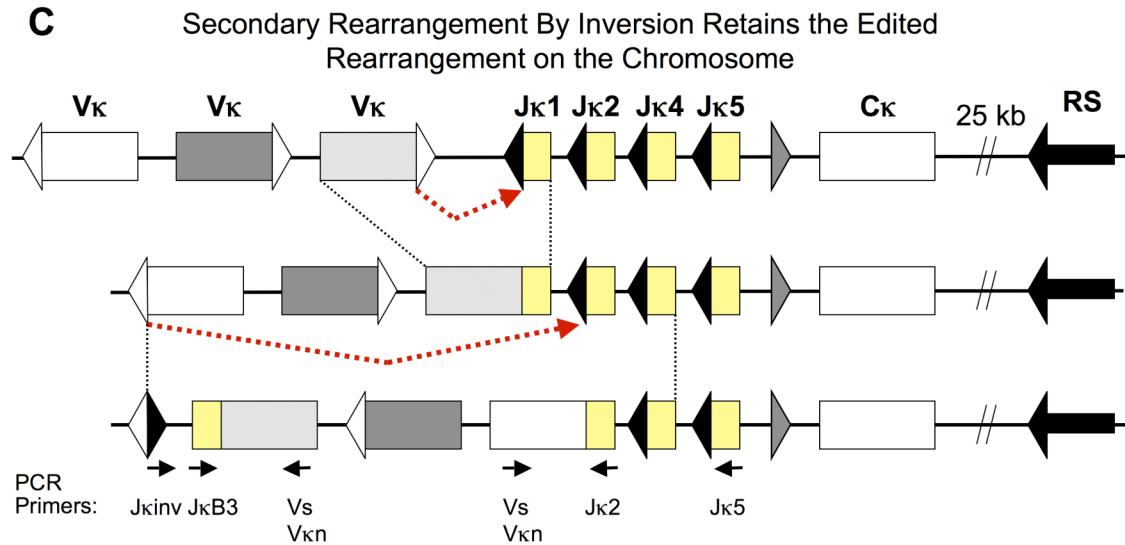
**Figure 3-1**



**Figure 3-1: RS rearrangements are increased in B cells expressing an autoreactive heavy chain in self-tolerant mice.** (a) Flow cytometry of splenic follicular (B220<sup>+</sup>, AA4.1<sup>-</sup>, CD23<sup>+</sup>) κ<sup>+</sup> B cells from heterozygous B6.56R mice depicting separation of B cells by immunoglobulin heavy chain allotype. (b) Vκ-RS rearrangement frequencies measured in follicular B cells expressing endogenous heavy chains (IgM<sup>b</sup>+, black bar) and the 56R anti-DNA heavy chain (IgM<sup>a</sup>+, gray bar) from heterozygous B6.56R mice (n=4). All PCR reactions were performed in duplicate. Data are presented as fold difference relative to the average RS level in C57Bl/6 splenic B220<sup>+</sup> IgM<sup>+</sup> κ<sup>+</sup> B cells (dashed line). Error bars indicate +s.e.m. and the \* denotes p<0.05 by one-tailed Student's t-test. A one-tailed statistical test was chosen because an active model of receptor editing predicts an *increase* in RS rearrangement levels among 56R expressing B cells, while a passive model predicts no difference between groups (see main text).

**Figure 3-2**





**Figure 3-2: Multiple editing rearrangement pathways are possible at the murine Igκ locus.** Shown are idealized murine Igκ loci with 3 Vκ segments, the four functional Jκ segments, Cκ, the intronic recombination signal sequence (iRS) and the RS downstream of Cκ. Exons are denoted with boxes, introns with lines and recombination signal sequences (RSS) by triangles. The open triangles denote RSSs with 12 bp spacers and the shaded RSSs with 23 bp spacers. Dashed lines denote the rearranging gene segments. Small arrows denote primers used in RS and Vκ-Jκ genotyping assays. Figure (a) depicts deletional rearrangement generating an excision product. When deletional rearrangement occurs, the intervening DNA is lost on an episome (represented by a circle). Figure (b) represents deletional rearrangement involving the iRS and RS results in an inactivated Igκ allele (Cκ is released on an episome). This inactivated allele retains a Vκ-Jκ precursor rearrangement that can be analyzed by Vκ-RS PCR. Figure (c) portrays secondary rearrangement by inversion which retains the precursor rearrangement on the chromosome. Inversions can be typed by PCR by scoring for rearrangements that amplify with a proximal Jκ primer but fail to amplify with a distal Jκ or Cκ primer.



**Figure 3-3**

Strain	Mouse/ Fusion Germline	Clone#	V	-----IRS1-----   JXN   -----RS-----
				TTTCCTGAGGACACAGTG CACTGTGAGGACCCTAGT
3H9/56R+/+	1	B.B.1	Vk38c	TTTCC CTAGT
3H9/56R+/+	2	C.A.2	Vk38c	TTTCCTGAGG C GGACCCTAGT
3H9/56R+/+	5	D.B	Vk38c	TTTCCTGAGG CCCTAGT
3H9+/+	1	B.D.1	12-44	TTTCCTGA CCCTAGT
3H9+/+	2	A.C.2	12-44	TTTCT GGACCCTAGT
3H9+/+	2	A.C.3	12-44	TTTCCTGA CCCTAGT
3H9+/+	1	B.B.2	12-46	TTTCCTG GACCCTAGT
3H9+/+	2	A.B.2	12-46	TTTCCTGA CCCTAGT
3H9+/+	5	A.A.1	12-46	TTT CCCTAGT
3H9/56R+/+	1	B.2	12-44	TTTCCTGA CCCTAGT
3H9/56R+/+	3	C.2	12-44	TTTCC AGT
3H9/56R+/+	1	B.2	12-46	TTTCCTG AGT
3H9/56R+/+	4	C.1	12-46	TTTCCTGAGG CCT CTAGT
3H9/56R+/+	6	C.1	12-46	TTTCCC ACCCTAGT
3H9/56R+/+	6	C.3	12-46	TTTCCTGA CCCTAGT
56R+	LPS	680.37.1	kn4	TTTCCTGAGG C CCCTAGT
56R+	LPS	680.39.3	21-12	TTTCCTG TAGT
56R+	Spon	680SP561	hf24	TTTCC GACCCTAGT
56R+	Spon	680SP603	21-3	TTTCCTGAGG CCCTAGT
56R+BclXL+	LPS	972.56C	aq4/kk4	TTTCC GGACCCTAGT
56R+BclXL+	LPS	972.821	aj4	TTTCCT GACCCTAGT

**Figure 3-3: iRS-RS junctions demonstrate a high degree of diversity.** iRS-RS rearrangements were cloned and sequenced from genomic spleen DNA of 56R<sup>+/+</sup>, 3H9<sup>+/+</sup> mice and from 56R<sup>+</sup> hybridomas. Mouse or clone numbers are listed along with the identity of V<sub>κ</sub> genes edited by iRS-RS rearrangement. RS junctional deletions are aligned relative to the junction predicted by RSS sequences. Nucleotides attributable to either iRS or RS are listed in the middle.

**Figure 3-4**

a

56R Vk38c-Jk5

Strain	Mouse#	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
			Vk38c	CTACAGTATGATAATCTTCTA		CTCACGTTTCGGTGCT	
				L Q Y D N L L		L T F G A	
3H9/56R+/+	741	1.3	Vk38c	CTACAGTATGATAATC		CTCACGTTTCGGTGCT	O/F
				L Q Y D N P		H V R C	
3H9/56R+/+	732	1.3	Vk38c	CTACAGTATGATAATCT		CTCACGTTTCGGTGCT	O/F
				L Q Y D N L		S R S V	
3H9/56R+/+	732	5.3	Vk38c	CTACAGTATGATAATCTTCTA		CTCACGTTTCGGTGCT	I/F
				L Q Y D N L L		L T F G A	
3H9/56R+/+	1	C	Vk38c	CTACAGTATGATAATCTTC	G	CTCACGTTTCGGTGCT	O/F
				L Q Y D N L R		S R S V	
3H9/56R+/+	1	B.B.1	Vk38c	CTACAGTATGATAATCTT		CTCACGTTTCGGTGCT	I/F
				L Q Y D N L		L T F G A	
3H9/56R+/+	2	C.A.2	Vk38c	CTACAGTATGATAATCTT		TCACGTTTCGGTGCT	O/F
				L Q Y D N L		S R S V	
3H9/56R+/+	3	C.A.1	Vk38c	CTACAGTATGATAATCTTC	G	CTCACGTTTCGGTGCT	O/F
				L Q Y D N L R		S R S V	
3H9/56R+/+	4	C.A.2	Vk38c	CTACAGTATGATAATCTTCT	G	CTCACGTTTCGGTGCT	I/F
				L Q Y D N L L		L T F G A	
3H9/56R+/+	4	C.C	Vk38c	CTACAGTATGATAATCTTCTA		CACGTTTCGGTGCT	O/F
				L Q Y D N L L		H V R C	
3H9/56R+/+	5	D.A.2	Vk38c	CTACAGTATGATAATCTTCTA		CCACGTTTCGGTGCT	O/F
				L Q Y D N L L		P R S V	
3H9/56R+/+	5	D.B	Vk38c	CTACAGTATGATAATC	G	CTCACGTTTCGGTGCT	O/F
				L Q Y D N R		S R S V	

**b****3H9 Vκ12/13-Jκ5**

Strain	Mouse	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
	Germline		12-44	CAACATCATTATGGTACTCCT		CTCACGTTTCGGTGCT	
				Q H H Y G T P		L T F G A	
3H9+/+	1	B.D.1	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9+/+	1	C	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9+/+	2	A.C.1	12-44	CAACATCATTATGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H H Y G T P		H V R C	
3H9+/+	2	A.C.2	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9+/+	2	A.C.3	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9+/+	2	C.C.1	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9+/+	4	A.A.3	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
Strain	Mouse	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
	Germline		12-46	CAACATTTTTGGGTACTCCT		CTCACGTTTCGGTGCT	
				Q H F W G T P		L T F G A	
3H9+/+	1	B.B.2	12-46	CAACATTTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9+/+	1	C.D.3	12-46	CAACATTTTTGGGGTACTCCT		CACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9+/+	1	C.D.3	12-46	CAACATTTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9+/+	2	A.B.2	12-46	CAACATTTTTGGGGTACTCC	G	CTCACGTTTCGGTGCT	I/F
				Q H F W G T P		L T F G A	
3H9+/+	2	A.D.2	12-46	CAACATTTTTGGGGTACTCC		CGTTTCGGTGCT	O/F
				Q H F W G T P		V R C	
3H9+/+	2	C.A.3	12-46	CAACATTTTTGGGGTACTCCGAGCG		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P S A		H V R C	
3H9+/+	2	C.B.3	12-46	CAACATTTTTGGGGTACTCCT	G	CTCACGTTTCGGTGCT	O/F
				Q H F W G T P	A	H V R C	
3H9+/+	3	A.C.1	12-46	CAACATTTTTGGGGTACT	G	CTCACGTTTCGGTGCT	O/F
				Q H F W G T	S	H V R C	
3H9+/+	5	A.A.1	12-46	CAACATTTTTGGGGTACTCC	G	CTCACGTTTCGGTGCT	I/F
				Q H F W G T P		L T F G A	
3H9+/+	5	E	12-46	CAACATTTTTGGGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H F W G T P	P	R S V	

**c****56R Vk12/13-Jk5**

Strain	Mouse	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
	Germline		12-44	CAACATCATTATGGTACTCCT		CTCACGTTTCGGTGCT	
				Q H H Y G T P		L T F G A	
3H9/56R+/+	1	B.2	12-44	CAACATCATTATGGTACTCCT	C	CACGTTTCGGTGCT	O/F
				Q H H Y G T P	P	R S V	
3H9/56R+/+	3	C.2	12-44	CAACATCATTATGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H H Y G T P		H V R C	

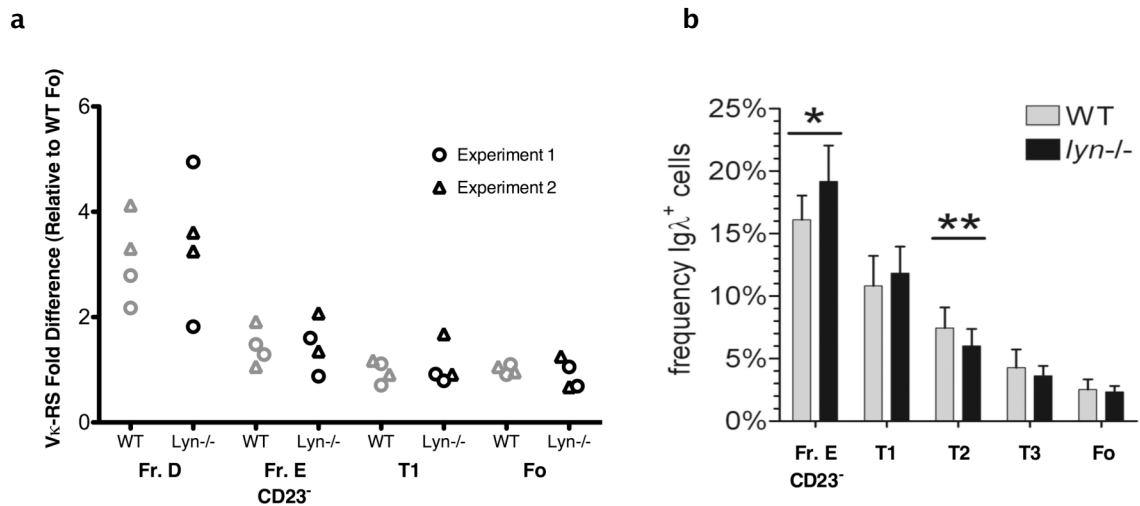
Strain	Mouse	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
	Germline		12-46	CAACATTTTGGGGTACTCCT		CTCACGTTTCGGTGCT	
				Q H F W G T P		L T F G A	
3H9/56R+/+	1	B.2	12-46	CAACATTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9/56R+/+	1	D.2	12-46	CAACATTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9/56R+/+	2	A.1	12-46	CAACATTTTGGGGTACTCCG		CTCACGTTTCGGTGCT	I/F
				Q H F W G T P		L T F G A	
3H9/56R+/+	3	B.2	12-46	CAACATTTTGGGGTACTGCG		CTCACGTTTCGGTGCT	I/F
				Q H F W G T A		L T F G A	
3H9/56R+/+	4	C.1	12-46	CAACATTTTGGGGTACTC	C	CGTTCGGTGCT	O/F
				Q H F W G T P		V R C	
3H9/56R+/+	4	C.2	12-46	CAACATTTTGGGGTACTC	C	CGTTCGGTGCT	O/F
				Q H F W G T P		V R C	
3H9/56R+/+	5	C.3	12-46	CAACATTTTGGGGTACCT		TCACGTTTCGGTGCT	I/F
				Q H F W G T F		T F G A	
3H9/56R+/+	6	C.1	12-46	CAACATTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9/56R+/+	6	C.3	12-46	CAACATTTTGGGGTACT		CTCACGTTTCGGTGCT	I/F
				Q H F W G T		L T F G A	
3H9/56R+/+	8	B.2	12-46	CAACATTTTGGGGTACTC		CTCACGTTTCGGTGCT	O/F
				Q H F W G T P		H V R C	
3H9/56R+/+	8	D.1	12-46	CAACATTTTGGGGTACTCCT		CTCACGTTTCGGTGCT	I/F
				Q H F W G T P		L T F G A	

**d****56R Hybridoma Vsh-Jk5**

Genotype	Fusion	Clone#	V	-----Vk-----	JXN	-----Jk5-----	Frame
		Germline	kn4	CATCAGCGGAGTAGTTACCCA		CTCACGTTTCGGTGCT	
				H Q R S S Y P		L T F G A	
56R+	LPS	680.37.1	kn4	CATCAGCGGAGTAGTT		CTCACGTTTCGGTGCT	O/F
				H Q R S S S		H V R C	
		Germline	21-12	CAGCACAGTAGGGAGCTTCCT	C	CTCACGTTTCGGTGCT	
				Q H S R E L P	P	H V R C	
56R+	LPS	680.39.3	21-12	CAGCACAGTAGGGAGCTTCCGAGCG		CTCACGTTTCGGTGCT	O/F
				Q H S R E L P S A		H V R C	
		Germline	8-24	CAGCAACATTATAGCACTCCT		CTCACGTTTCGGTGCT	
				Q Q H Y S T P		L T F G A	
56R+	LPS	680.52A	8-24	CAGCAACATTATAGCACTCCT		CTCACGTTTCGGTGCT	I/F
				Q Q H Y S T P		L T F G A	
		Germline	hf24	ATGCAACATCTAGAATATCCT		CTCACGTTTCGGTGCT	
				M Q H L E Y P		L T F G A	
56R+	Spon	680SP561	hf24	ATGCAACATCTAGAATATCC	G	CTCACGTTTCGGTGCT	I/F
				M Q H L E Y P		L T F G A	
		Germline	21-3	AGCAAAGTATTGAGGATCCTC		CTCACGTTTCGGTGCT	
				Q Q S I E D P		L T F G A	
56R+	Spon	680SP603	21-3	AGCAAAGTATTGAGGATC		CTCACGTTTCGGTGCT	I/F
				Q Q S I E D		L T F G A	
		Germline	23-48	CAACAAAGTAATAGCTGGCCA		CTCACGTTTCGGTGCT	
				Q Q S N S W P		L T F G A	
56R+	LPS	680.103.2	23-48	CAACAAAGTAATAGCTGGCCA	ACT	ACGTTTCGGTGCT	I/F
				Q Q S N S W P	T	T F G A	
		Germline	aq4/kk4	CAGTGGAGTAGTAACCCACCC		CTCACGTTTCGGTGCT	
				Q W S S N P P		L T F G A	
56R+	LPS	680.56.1	aq4/kk4	CAGTGGAGTAGTAACCCA	G	CTCACGTTTCGGTGCT	O/F
				Q W S S N P	A	H V R C	
56R+BclXL+	LPS	972.30A	aq4/kk4	CAGTGGAGTAGTAACCC	G	CTCACGTTTCGGTGCT	I/F
				Q W S S N P		L T F G A	
56R+BclXL+	LPS	972.56C	aq4/kk4	CAGTGGAGTAGTAACCCA	G	CTCACGTTTCGGTGCT	O/F
				Q W S S N P	A	H V R C	
		Germline	aj4	CAGTGGAGTAGTTCCCCACCC		CTCACGTTTCGGTGCT	
				Q W S S S P P		L T F G A	
56R+BclXL+	LPS	972.821	aj4	CAGTGGAGTAGTTCCCCACCC	G	CTCACGTTTCGGTGCT	O/F
				Q W S S S P P	A	H V R C	

**Figure 3-4: A high frequency of editing precursors on RS deleted kappa alleles are in-frame.** Genomic DNA from 56R<sup>+/+</sup> or 3H9<sup>+/+</sup> mouse spleens (a-c) or from hybridomas (d) was subjected to PCR using primers situated in V<sub>κ</sub> (38c, 12 or Vs) and RS (see materials and methods). Amplicons over 1.2kb in size were cloned and sequenced in both directions. Figures (a-c) show the mouse genotype, animal number, V<sub>κ</sub>-J<sub>κ</sub> junction and whether the junction is in frame (I/F) or out of frame (O/F). Figure (d) shows the V<sub>κ</sub>-J<sub>κ</sub>5 rearrangements recovered from RS deleted alleles in 56R<sup>+</sup> LPS and spontaneously fused hybridomas.

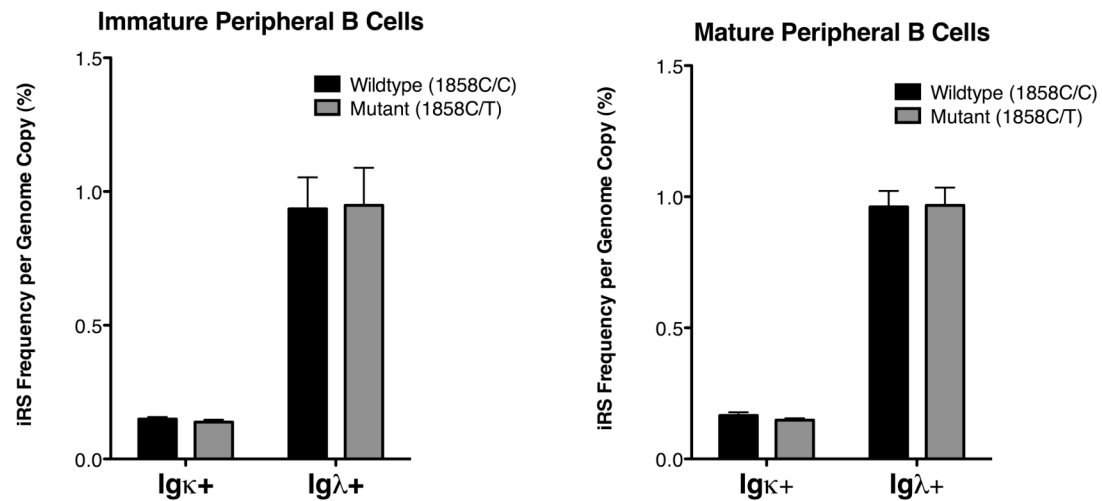
**Figure 3-5**



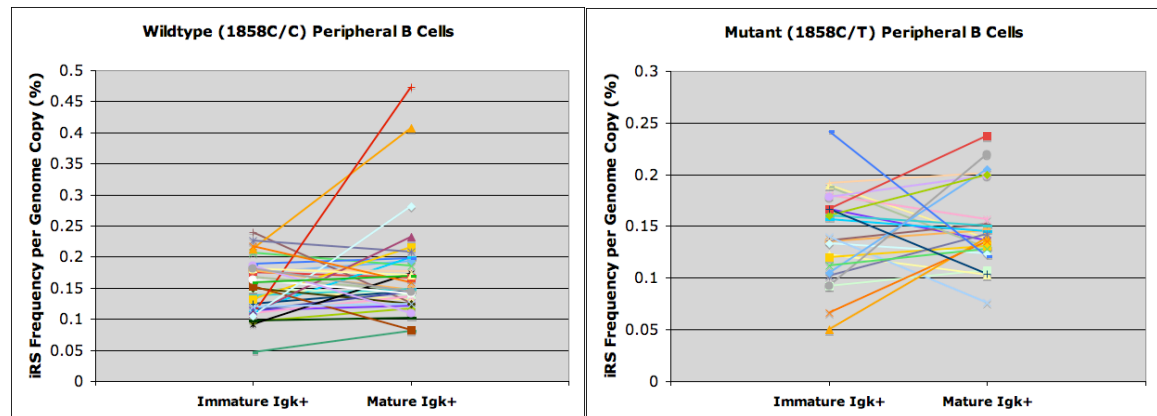
**Figure 3-5: Absence of Lyn does not impair receptor editing and clonal deletion of immature B cells.** (a) V $\kappa$ -RS rearrangement levels were measured in genomic DNA from Ig $\lambda$ <sup>-</sup> bone marrow (Fr. D and Fr. E) and splenic (T1 and Fo) B cell populations from Lyn<sup>-/-</sup> and wild-type (WT) mice. Two mice were analyzed per experiment and results from two separate experiments are depicted. All PCR reactions were performed in duplicate. Data are represented as fold difference relative to the mean RS level in wild-type Ig $\lambda$ <sup>-</sup> follicular B cells. (b) The frequency of Ig $\lambda$ <sup>+</sup> B cells in bone marrow (Fr. E) and splenic (T1, T2, T3, Fo) B cell populations from Lyn<sup>-/-</sup> (n=10) and wild-type (n=11) as assessed by flow cytometry. Data are represented as mean+SD (\*, p=0.01; \*\*, p=0.04).

**Figure 3-6**

**a**



**b**

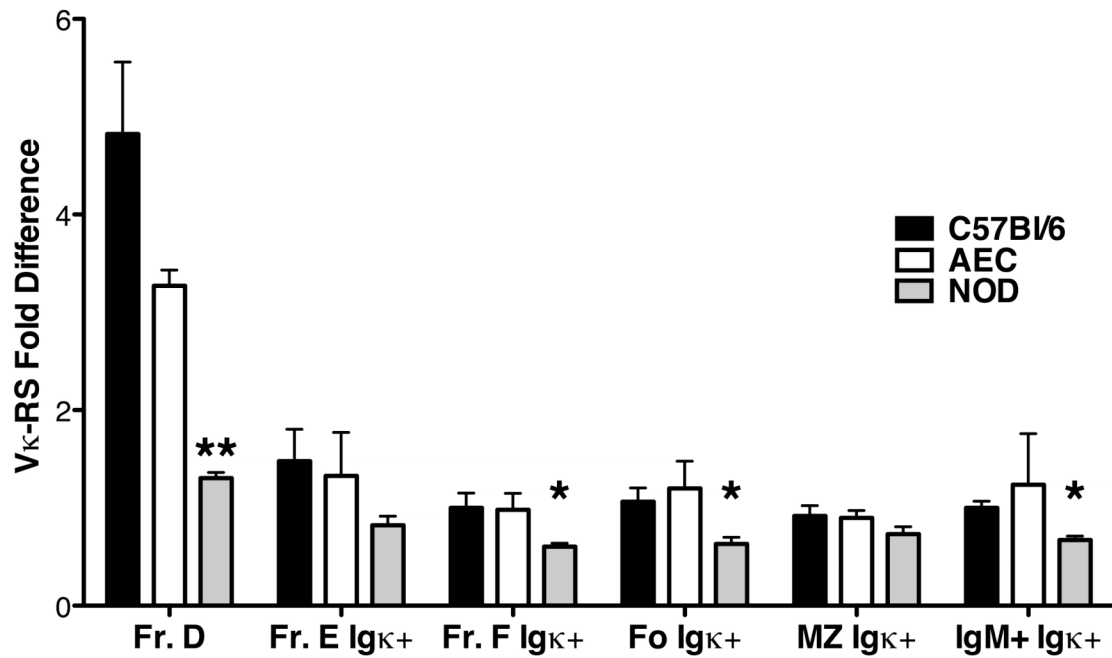


**Figure 3-6: The PTPN22 1858T variant does not affect receptor editing in healthy individuals.** (a) iRS rearrangement frequencies as quantified in genomic DNA from peripheral immature (CD19<sup>+</sup> CD24<sup>hi</sup> CD38<sup>hi</sup>, left) and mature (CD19<sup>+</sup> CD24<sup>low</sup> CD38<sup>int</sup>, right) B cells of healthy individuals with wild-type or mutant (1858C/T) PTPN22. For immature Igκ<sup>+</sup> B cells, n=36 for WT, n=27 for mutant. For mature Igκ<sup>+</sup> B cells, n=39 for WT, n=30 for mutant. Among immature Igλ<sup>+</sup> B cells, n=16 for WT, n=12 for mutant. Among mature Igλ<sup>+</sup> B cells, n= 18 for WT and n=17 for mutant. All PCR

reactions were performed in duplicate. (b) Differences in iRS rearrangement frequencies between immature and mature Igκ<sup>+</sup> B cells tracked per individual. Values from individuals with WT PTPN22 are depicted on the left (n=35) and those with mutant PTPN22 on the right (n=25).



**Figure 3-7**



**Figure 3-7: AEC mice display RS rearrangement levels equivalent to wild-type mice.**  $V_{\kappa}$ -RS rearrangement levels in genomic DNA from bone marrow (Fr. D, Fr. E, and Fr. F) and splenic (Fo, MZ, and IgM<sup>+</sup>) B cells of AEC mice (n=2, white bars). Data from C57Bl/6 (black bars) and NOD (gray bars) mice are plotted for comparison. All PCR reactions were performed in duplicate. Data represent fold difference relative to the mean RS level in C57Bl/6 B220<sup>+</sup> IgM<sup>+</sup> Igκ<sup>+</sup> splenic B cells (+SEM; \* = p<0.05; \*\* = p<0.01).

**Table 3-1**

Rearrangement Model	Precursor IF %	IF Precursor Repertoire
Prompt and Irreversible H/L-STOP	0%	Not applicable
“Leaky” H/L-STOP	33%	Unrestricted
Classical Editing	33% > ... > 0% Upper limit not defined*	Autoreactive

**Table 1: Three models of receptor editing predict different frequencies of in-frame editing precursors.** In a model of complete feedback inhibition (prompt and irreversible H/L STOP), IgL rearrangement ceases with the expression of a function BCR on the cell surface irrespective of specificity. Consequently, this model predicts that no editing precursors should be in-frame (IF). In a model with incomplete (or the total absence of) feedback inhibition, IgL rearrangement may continue despite BCR surface expression. Assuming IgL recombination results in an in-frame rearrangement 1/3 of the time, this model would predict 33% of editing precursors to be in-frame. Moreover, if continued IgL rearrangement occurs without regard to BCR specificity, the specificity of IF editing precursors would be unbiased. The classical model of receptor editing proposes that receptor editing occurs in response to autoreactivity. Based upon this model up to 33% of editing precursors would be predicted to be IF, however, this model also predicts that IF precursors should encode for autoreactive light chains. \*Of note, the upper limit of IF frequency is variable, as it may be increased with positive selection for autoreactive receptors before editing or decreased with negative selection of B cells with autoreactive receptors prior to editing.

**Table 3-2**

Fusion Genotype	Clone	Editor V $\kappa$ /J $\kappa$	Inverted V $\kappa$ /J $\kappa$ 1 (RP)	RP V $\kappa$ Seq.	J $\kappa$ 1 Seq.	Frame
3H9/GL J $\kappa$ <sup>+/-</sup>	24	12/J2	19-25	agc act cct	acg ttc ggt	OF
	26	12/J2	12-41	agt act	tgg acg ttc ggt	OF
	60	12/J2	gr32	agt tat cct	tgg acg ttc ggt	OF
	100	12/J2	19-17	agt act cct	acg ttc ggt	IF
3H9 J $\kappa$ <sup>+/-</sup>	7	12/J2	bd2	cat ttt	tgg acg ttc ggt	IF
	25	12/J5	21-3	gag gat cct	acg ttc ggt	IF
	26	12/J2	21-2	gag gtt	acg ttc ggt	OF
	37	12/J5	kb4	tat cct ctt	acg ttc ggt	OF
	79	12/J2	8-24	agc act	tgg acg ttc ggt	IF
56R J $\kappa$ <sup>+/-</sup>	26	21D/J4	aq4	aac cca	acg ttc ggt	OF
	40	21D/J2	8-19	agt tat	tgg acg ttc ggt	OF
	49	21D/J2	8-21	tat aat ctt	acg ttc ggt	IF
	96	21D/J2	19-15	agc tat cct	acg ttc ggt	OF

**Table 2: Nucleotide sequences at the junctions of inverted V $\kappa$ -J $\kappa$ 1 rearrangements reveal several IF editing precursors.** Clones with J $\kappa$  inversions are defined as described in the text. 13 reciprocal products (RP) are presented, which were isolated from hybridomas derived from three different IgH transgenic strains hemizygous for J $\kappa$ -C $\kappa$  deletion. Listed are the hybridoma genotypes, clone number, editor V $\kappa$  gene expressed, and the identity of the V $\kappa$  gene present in the RP. All the Vs/J $\kappa$ 1inv PCR products were sequenced to determine whether the RPs were in-frame (IF) or out-of-frame (OF).

## **CHAPTER 4: Summary and Future Directions**

## SUMMARY

In these studies I explored the role of receptor editing in the context of autoimmune disease and investigated the stimuli responsible for promoting continued immunoglobulin light chain (IgL) recombination. In Chapter 2, I described the development of a novel assay for monitoring tolerance in B cell populations. The RS rearrangement assay measures a late receptor editing event involving the recombination of a non-coding gene segment in the Ig $\kappa$  locus, RS or recombining sequence, that results in the functional inactivation of Ig $\kappa$ . Since RS does not code for a constituent of the BCR (Daitch et al. 1992), the RS rearrangement assay is not restricted to certain autoantigens and thus is broadly applicable to many autoimmune diseases. Additionally, the RS gene segment is also found in humans (called the kappa deleting element, KDE) (Siminovitch et al. 1985), thus it was easy to adapt the assay for use in human studies.

Applying this assay I observed that B cells from mouse models of SLE (MRL/lpr) and T1D (NOD) harbored fewer RS rearrangements than wild-type mice (C57Bl/6), suggesting that defects in receptor editing may contribute to autoimmune disease. Interestingly, related mouse strains that develop less severe autoimmunity (MRL<sup>+/+</sup>, NOR) also showed evidence of decreased RS recombination, indicating that impaired receptor editing likely increases disease susceptibility but additional factors are involved in the development of more severe disease. Additionally, the RS assay was used to characterize B cells from patients with SLE and T1D. Approximately 30% of people from either disease group had lower RS levels than the 10<sup>th</sup> percentile of healthy individuals. This finding suggests that defects in receptor editing may also contribute to autoimmune disease in people. Moreover, since some patients with autoimmune disease had relatively normal RS levels, the results also indicate heterogeneity among patients. As the RS rearrangement assay measures the dominant mechanism of central

tolerance, this variability suggests differences in the efficacy of central tolerance in autoimmune patients, which may be relevant in treatment decisions.

In Chapter 3 I explored the forces that may control antibody light chain receptor editing, including RS rearrangement. Although increasing evidence suggests that receptor editing occurs in response to BCR autoreactivity of immature B cells (Hertz and Nemazee 1997; Halverson et al. 2004; Tze et al. 2005), conclusive demonstration of receptor editing induced by positive BCR signaling *in vivo* has been lacking. Using IgH transgenic mice, I sought to correlate B cell autoreactivity with receptor editing. Indeed, B cells expressing the anti-DNA IgH 56R underwent RS rearrangement more often than those expressing a wild-type IgH. This mechanism was further explored through the characterization of editing precursors. If autoreactivity stimulates receptor editing, then functional (in-frame) IgL rearrangements that have been edited should encode autoreactive light chains. Analysis of editing precursors generated through RS and inversional V $\kappa$  rearrangement revealed a bias toward autoreactive light chains in multiple transgenic mouse strains, consistent with an autoreactivity-driven model of receptor editing.

To further investigate if self-antigen induced positive signaling promotes receptor editing, I assessed the role of two BCR signaling components in RS recombination. I found that lack of the inhibitory tyrosine kinase Lyn did not alter RS rearrangement levels. Similarly, in human B cells a dysregulated isoform of the lymphoid phosphatase PTPN22, which is associated with autoimmune diseases including SLE and T1D (Bottini et al. 2004; Lee et al. 2007; Harley et al. 2008), did not adversely affect receptor editing. Together these findings suggest that perturbation of these BCR signaling components alone do not affect receptor editing. Additionally, to identify other factors that may be responsible for decreased RS rearrangement observed in the context of autoimmunity, receptor editing was examined in a mouse model of Sjögren's

syndrome (AEC). This mouse strain carries two of over twenty genetic loci correlated with type 1 diabetes in the NOD mouse. However, unlike NOD mice, the AEC strain displayed RS rearrangement levels equivalent to wild-type mice, indicating that genes present on the two disease associated loci (*Idd3* and 5) alone do not influence receptor editing. Yet, this finding does not rule out the possibility that these loci act in concert with additional disease associated genes to influence receptor editing.

## **FUTURE DIRECTIONS**

### **Development of RS Rearrangement as a Clinical Assay**

As described in Chapter 2, decreased RS rearrangement frequencies were detected in approximately 30% of patients with SLE and T1D suggesting that low RS levels may have prognostic value in determining the risk of developing autoimmune disease. However several issues need to be addressed before the RS assay can be applied in this manner. First, we need to determine how reproducible RS rearrangement levels are in individuals. It may be possible that RS levels vary at different stages of autoimmune disease. If B cells lacking RS rearrangements are involved in the pathogenesis of autoimmunity, these cells may be more prevalent during the early stages of disease process, possibly before the appearance of symptoms, as well as during disease flares. Therefore it would be informative to measure RS levels at multiple time points in healthy individuals to define a baseline level of variability and then determine if RS levels change more in patients with autoimmune disease. If RS rearrangement levels are found to be consistently lower in patients with autoimmune disease, it may be feasible to use the assay to predict an increased risk of developing autoimmune disease in undiagnosed individuals. Studies comparing RS levels of siblings without disease to those with autoimmunity will also allow us to determine how closely RS levels correlate with autoimmune disease. Furthermore, prospective studies

of individuals with average and low RS rearrangement levels would help define the relative risk of developing autoimmunity that low RS levels confer and demonstrate the possible prognostic value of the assay. Finally, it would be interesting to see if RS rearrangement levels are similarly decreased in other autoimmune diseases such as rheumatoid arthritis and myasthenia gravis. As the RS rearrangement assay is not dependent upon a specific autoantigen, it could easily be applied to assess central tolerance in these diseases.

In addition to prognostic applications, the RS rearrangement assay may also be useful as a diagnostic tool. As not all patients with autoimmune disease demonstrated decreased RS rearrangement frequencies, the RS assay has revealed heterogeneity among SLE and T1D patients that may have implications in treatment decisions. Decreased RS levels are suggestive of defects in central B cell tolerance, which is relevant to treatment since current therapies for SLE have included B cell depletion with rituximab (anti-CD20 mAb) (Leandro et al. 2002; Eisenberg 2006). Rituximab is also being considered in immunosuppressive regimens used after islet cell transplant in T1D patients. The premise behind use of this antibody in autoimmune disease treatment is to deplete the majority of circulating B cells (those that express CD20) with the hope that autoreactive B cells will also be eliminated and lead to remission or decreased severity of disease. However, if a subset of patients generate autoreactive B cells due to defects in central tolerance, it is likely that such B cells will be generated again as the B cell repertoire is being reconstituted. Moreover, under conditions of lymphopenia developing B cells may experience greater effective concentrations of pro-survival factors such as B lymphocyte stimulator (BLyS, also known as BAFF) that could result in impaired deletion of autoreactive B cells (Lesley et al. 2004; Thien et al. 2004). Indeed, mice overexpressing BLyS develop lymphocytic expansions that share characteristics of autoimmune disease (Mackay et al. 1999). Additionally, patients with SLE, rheumatoid



arthritis (RA), and Sjögren's syndrome have been shown to have increased serum levels of BLyS (Cheema et al. 2001; Zhang et al. 2001). Thus, B cell depletion in patients with central tolerance defects may in fact worsen their disease as the accompanying lymphopenia could foster the development and activation of newly generated autoreactive B cells. Therefore the RS rearrangement assay may be useful in screening patients for defects in central tolerance and guiding their treatment.

### **RS Rearrangement Assay Optimization**

Although it can be performed in less than 2 days, in its current form the RS rearrangement assay is not amenable to clinical use, as it requires FACS isolation of B cells and their separation based upon light chain isotype. This separation is required due to the large differences in RS rearrangement levels between  $Ig\kappa^+$  and  $Ig\lambda^+$  B cells. However, if the RS levels could be reliably measured from whole blood genomic DNA it would shorten the time required and forego the need for FACS B cell isolation making the assay clinically viable. Measuring RS rearrangements in whole blood is not completely straightforward, however, as inter-individual differences in the frequency of B cells and the  $\kappa:\lambda$  ratio can shift overall RS rearrangement levels significantly. To compensate for this variability aliquots of blood samples could be analyzed by flow cytometry along with RS analysis to determine the B cell frequency and  $\kappa:\lambda$  ratio. This information could then be used to calculate an individualized correction factor allowing RS levels to be compared between individuals.

One caveat to this approach is that correction for the  $\kappa:\lambda$  ratio requires an assumed value of RS rearrangement in  $Ig\kappa^+$  and  $Ig\lambda^+$  B cells. As we saw in Chapter 2, these values differ among individuals and more importantly they do not always change to the same degree. For instance, an individual may have an average level of RS rearrangement among  $Ig\kappa^+$  B cells, while  $Ig\lambda^+$  B cells have lower than average RS levels.

One possibility to circumvent this problem would be to only focus on B cells of a single light chain isotype. Given the increased RS rearrangement values in  $\text{Ig}\lambda^+$ , analyzing these cells would provide a greater dynamic range in which to detect differences.  $\text{Ig}\lambda^+$  B cells could be isolated from whole blood using magnetic bead separation, which may increase the time needed for RS analysis but still make it feasible in a clinical environment. We are currently in the process of determining if this approach can provide accurate and reproducible measurement of RS rearrangement levels.

### **Control of RS Recombination**

The studies described in this dissertation introduce a novel assay for estimating the degree of receptor editing in B cell populations through RS rearrangement levels, however, the mechanisms controlling RS recombination are unclear. Although autoreactivity appears to be correlated with RS rearrangement, it is possible that other factors independently regulate RS recombination. One possibility is that prolonged residence time of pre-B cells in the bone marrow leads to increased IgL rearrangement. Nussenzweig and colleagues observed that pre-B cells undergoing receptor editing experienced a 2-hour developmental delay (Casellas et al. 2001), which together with the finding that RS rearrangement occurs late in this process (Zou et al. 1993; Klein et al. 2005), suggests that B cells require increased time in the bone marrow to undergo extensive Ig $\kappa$  rearrangement. This process has been described in T cell development, where absence of the nuclear receptor ROR $\gamma$  (retinoic acid receptor-related orphan receptor) causes a decrease in the lifespan of double positive (DP) T cells and a concomitant decrease in TCR $\alpha$  rearrangement (Guo et al. 2002). Moreover, over-expression of the anti-apoptotic gene Bcl-x $_L$  increased DP T cell lifespan and promoted use of downstream 3' J $\alpha$  gene segments, indicating that a survival window influences TCR $\alpha$  rearrangement.

Correspondingly, if the level of RS rearrangement is related to the time pre-B cells spend in bone marrow, conditions that extend this time may result in increased RS levels. Indeed, expression of Bcl-x<sub>L</sub> in developing B cells increased RS rearrangement levels compared to wild-type, suggesting that a developmental window may function in a similar manner in B cells (**Fig. 4-1**). Bone marrow residence time may also provide an alternative explanation for the increased RS rearrangement observed among anti-DNA (56R<sup>+</sup>) B cells (**Fig. 3-1**). It may be possible that expression of the transgenic IgH leads to “premature entry” of early stage B cells into the pre-B cell compartment and, consequently, an extended window for IgL rearrangement. To distinguish if autoreactivity or increased bone marrow residence time are responsible for increased RS rearrangement, it would be informative to measure RS levels from other IgH transgenic B cells with non-autoreactive B cell receptors. Although the inability to screen for reactivity to all possible antigens makes determination of non-autoreactivity problematic, additional markers of non-autoreactivity, such as an unrestricted (wild-type) IgL repertoire and normal peripheral B cell counts, may facilitate this determination. Nevertheless, if these B cells also demonstrate higher RS levels, it would suggest that time in the bone marrow, rather than BCR specificity, controls RS rearrangement.

Exploration of the pre-BCR signaling pathway has revealed several factors that promote IgL rearrangement (**Fig. 1-2**), which also could be involved in the regulation of RS recombination. Recent reports have implicated the transcription factor Foxo1 in the upregulation of Rag required for IgL rearrangement (Amin and Schlissel 2008). Through this activity, Foxo1 may also influence RS recombination, as this event appears to occur late in the process of IgL rearrangement and thus would require extended expression of Rag. Nemazee and colleagues have proposed a model in which limiting levels of Rag in pre-B cells leads to the hierarchical pattern of IgL rearrangement, with Ig $\kappa$  rearranging

before RS and Ig $\lambda$  (Vela et al. 2008). They propose that this restriction leads to competition among Rag substrates and that RS rearrangement occurs less frequently than V $\kappa$  rearrangement due to its sub-optimal recombination recognition sequence (RSS). This non-canonical RSS may recruit Rag less efficiently and therefore compete poorly with V $\kappa$  gene segments that possess more favorable RSSs (Ramsden and Wu 1991). Thus, increased expression of Rag mediated by Foxo transcription factors may improve the chances of RS rearrangement. Conversely decreased expression of Foxo1 or its export from the nucleus due to Akt-mediated phosphorylation could lead to decreased RS recombination and in turn impaired receptor editing if functional Ig $\kappa$  chains cannot be silenced via RS rearrangement. Measuring Foxo1 expression or phosphorylation in pre-B and immature B cells of mouse strains demonstrating decreased RS recombination would be informative in this regard. If Foxo1 levels or activity are found to be correlated with RS recombination, the causality of this relationship could be explored by experimentally altering the expression of the transcription factor. Specifically, Foxo1 expression could be decreased in immature 56R<sup>+/+</sup> B cells to determine if this results in impaired editing and moreover, if this deficiency could cause a loss of tolerance and development of autoimmune disease. Foxo1 knockdown could be achieved by introduction of Foxo1 specific shRNA into primary bone marrow B cells via *ex vivo* retroviral transduction (as described in (Amin and Schlissel 2008)). Transduced B cells could then be introduced into irradiated hosts and RS rearrangement levels of the resulting mature peripheral B cells could be assessed. Additionally, markers of autoimmunity, such as increased serum antibody titers and histological changes could be examined.

Interferon regulatory factor 4 (IRF-4) has also been shown to be a critical mediator of pre-BCR signaling that serves to shut down pre-B cell proliferation and stimulate IgL rearrangement. IRF-4 promotes Ig $\kappa$  rearrangement directly through

interactions with the 3'E $\kappa$  (Eisenbeis et al. 1995), as well as indirectly by enhancing E2A binding of the iE $\kappa$  via downregulation of IL-7 signaling (Lazorchak et al. 2006; Johnson et al. 2008). Recently Pathak *et al.* reported that IRF-4 might also be required for efficient receptor editing in response to membrane antigens (Pathak et al. 2008). The authors found that IRF-4 deficient mice expressing a transgenic anti-HEL BCR failed to produce non-autoreactive B cells when exposed to membrane bound HEL (mHEL), suggesting that autoreactive B cells were largely deleted or anergized rather than tolerized via receptor editing. The authors confirmed that IRF-4<sup>-/-</sup> pre-B cells underwent less RS and  $\lambda$ 1 rearrangement compared to IRF-4 replete cells and found that IRF-4 transcription was induced by BCR ligation in immature B cells. Taken together these findings support a role for IRF-4 in receptor editing and suggest that deficiencies in IRF-4 activity may contribute to decreases in RS rearrangement levels observed in autoimmune individuals. Indeed, aberrant activity of IRF-4 in T cells is associated with development of rheumatoid arthritis and vasculitis (Chen et al. 2008). Thus measurement of IRF-4 expression and its binding levels at the Ig $\kappa$  enhancers as determined by chromatin immunoprecipitation (ChIP) in pre-B cells of MRL/lpr and NOD mice may begin to address whether IRF-4 contributes to the development of their autoimmune disease. Additionally, in concert with E2A, IRF-4 has been shown to increase histone acetylation and germline transcription of the Ig $\kappa$  locus (Lazorchak et al. 2006). Thus to further address potential aberrations in IRF-4 activity, histone acetylation at the Ig $\kappa$  locus can be examined by ChIP.

Upstream of IRF-4 and Foxo1 in the signaling cascade, the scaffold protein SLP-65 has also been implicated in stimulation of IgL rearrangement. Loss of SLP-65 results in decreased Ig $\kappa$  germline transcription and rearrangement in pre-B cells (Hayashi et al. 2003). More importantly, SLP-65 deficiency also impairs receptor editing, as Kitamura and colleagues observed that SLP-65<sup>-/-</sup> mice expressing an anti-DNA BCR (3H9/V $\kappa$ 4) fail

to rearrange endogenous  $V\kappa$  genes or RS and generate fewer  $Ig\lambda^+$  B cells (Hayashi et al. 2004). They also observed similar effects in mice with a wild-type BCR repertoire, indicating that SLP-65-mediated signaling is required for effective receptor editing. As SLP-65 activates several additional signaling molecules, it is unclear from these studies if SLP-65 directly promotes receptor editing or if it acts indirectly through its binding partners. Phospholipase  $C\gamma 2$  ( $PLC\gamma 2$ ) is one such signaling enzyme downstream of SLP-65 that has also been implicated in regulation of receptor editing (Wang et al. 2000; Bai et al. 2007). Wang and coworkers found that autoreactive B cells from  $PLC\gamma 2$  deficient mice did not receptor edit appropriately based upon decreased  $Ig\lambda$  surface expression and reduced endogenous  $Ig\kappa$  and  $Ig\lambda$  rearrangement. Furthermore, these cells demonstrated impaired IRF-4 upregulation in response to BCR stimulation. Together these findings suggest that SLP-65 or its binding partners may contribute to defects in receptor editing identified in Chapter 2. To clarify the role of this signaling pathway in MRL/lpr and NOD mice, the tyrosine phosphorylation status of these molecules in pre-B cells could be measured and compared to those from murine strains that receptor edit appropriately. Additionally, to test if impaired  $PLC\gamma 2$  activity is responsible for decreased editing, one could attempt to stimulate secondary rearrangements with ionomycin and phorbol 12-myristate 13-acetate (PMA) in order to activate protein kinase C (PKC), the main target of  $PLC\gamma 2$  activity.

### **The Significance of Altered RS Recombination**

As discussed in Chapter 2, two different mouse models of autoimmune disease as well as a subset of humans with SLE and T1D demonstrated decreased levels of RS rearrangement, indicating impairment of receptor editing. The commonality of decreased RS recombination among these individuals suggests that reduced editing may be a contributing factor to the development of autoimmune disease, however, the

consequence of the production of minimally edited cells remains unclear. An obvious question that arises from these findings is whether B cells lacking RS rearrangement tend to be more autoreactive. In Chapter 3, we saw that in self-tolerant mice, B cells expressing an anti-DNA heavy chain, 56R, underwent increased RS rearrangement compared to B cells expressing endogenous heavy chains, suggesting that BCR specificity influences the degree of RS rearrangement. Additionally, the prevailing model of receptor editing proposes that editing serves to mitigate autoreactivity of immature B cells so that peripheral B cells are largely non-autoreactive.

However, this is not always the case as receptor editing can also lead to light chain allelic inclusion (expression of two different functional light chains) and B cell polyreactivity (Liu et al. 2005; Witsch et al. 2006; Casellas et al. 2007). Due to intracellular antibody sequestration, IgL competition for IgH pairing, or partial autoreactivity (Liu et al. 2005; Doyle et al. 2006; Casellas et al. 2007), allelically included and polyreactive B cells may be able to bypass central tolerance mechanisms and participate in autoimmune responses. This scenario suggests that rather than abrogating autoreactivity, increased receptor editing may in fact promote development of autoimmune disease.

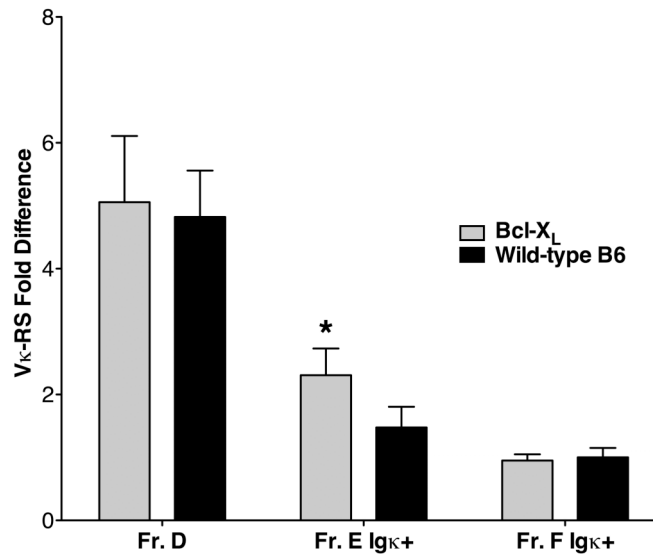
To determine whether B cells with increased or decreased editing levels participate in autoimmune responses, B cells specific for characteristic disease associated self-antigens could be examined. These studies could be facilitated through the isolation of antigen-specific B cells. For instance, serum anti-insulin antibodies are commonly found in patients with type-1 diabetes (Ziegler et al. 1991; Nakayama et al. 2005). Consequently, isolation and characterization of receptor editing levels of anti-insulin B cells may help determine the significance of decreased RS recombination in affected individuals. To determine if anti-insulin B cells could be identified by flow cytometry, I analyzed the insulin reactivity of 56R<sup>+/-</sup> splenocytes which have been shown

to recognize several auto-antigens, including insulin, based upon hybridoma studies of Ig $\kappa$  deficient 56R mice (Doyle et al. 2006). Preliminary studies using tetrameric insulin to identify anti-insulin B cells indicate that this approach may be feasible (**Fig. 4-2a**). Additionally, it is possible that frankly autoreactive B cells develop differently than poly/multi-reactive B cells. For example, autoreactive B cells may be generated with minimal receptor editing, while multireactive B cells may be allelically included secondary to extensive receptor editing. Therefore identifying multireactive B cells via double antigen staining will allow separate characterization of autoreactive and multireactive B cells (**Fig. 4-2b**).

If isolation of these B cells is possible, analysis of RS rearrangement levels and light chain allelic inclusion may provide evidence for different pathways of generating autoreactivity versus multireactivity. Moreover, if also performed in the context of autoimmune disease, it will allow for the assessment of the function of included and/or multireactive B cells in the disease process and clarification of the role of receptor editing. We may find that receptor editing is a double-edged sword, which as a whole functions to reduce autoreactivity, but can also provide a pathway for bypassing tolerance and generating self-reactive B cells critical for the development of autoimmunity.



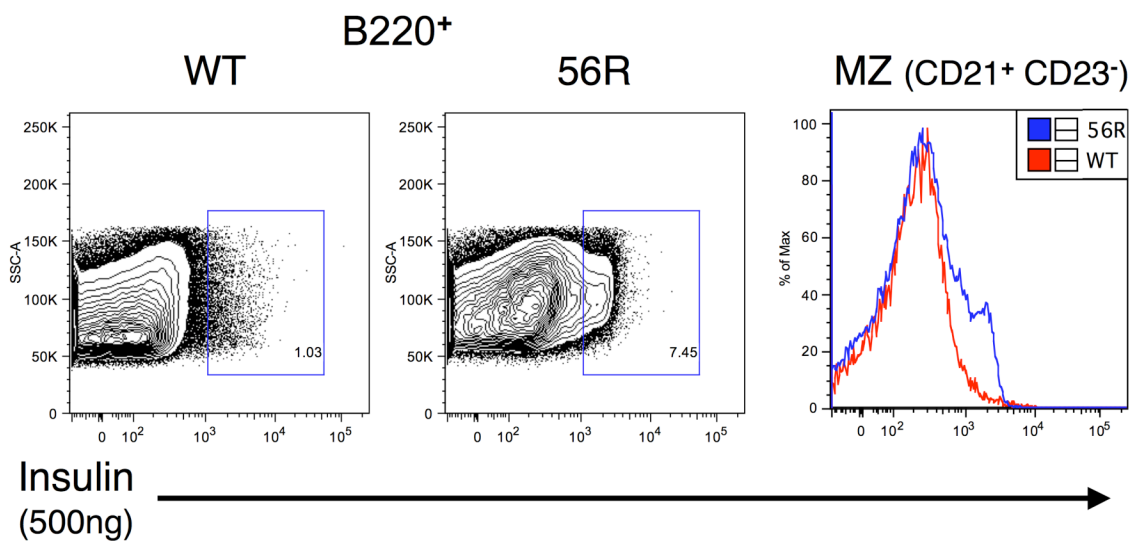
**Figure 4-1**



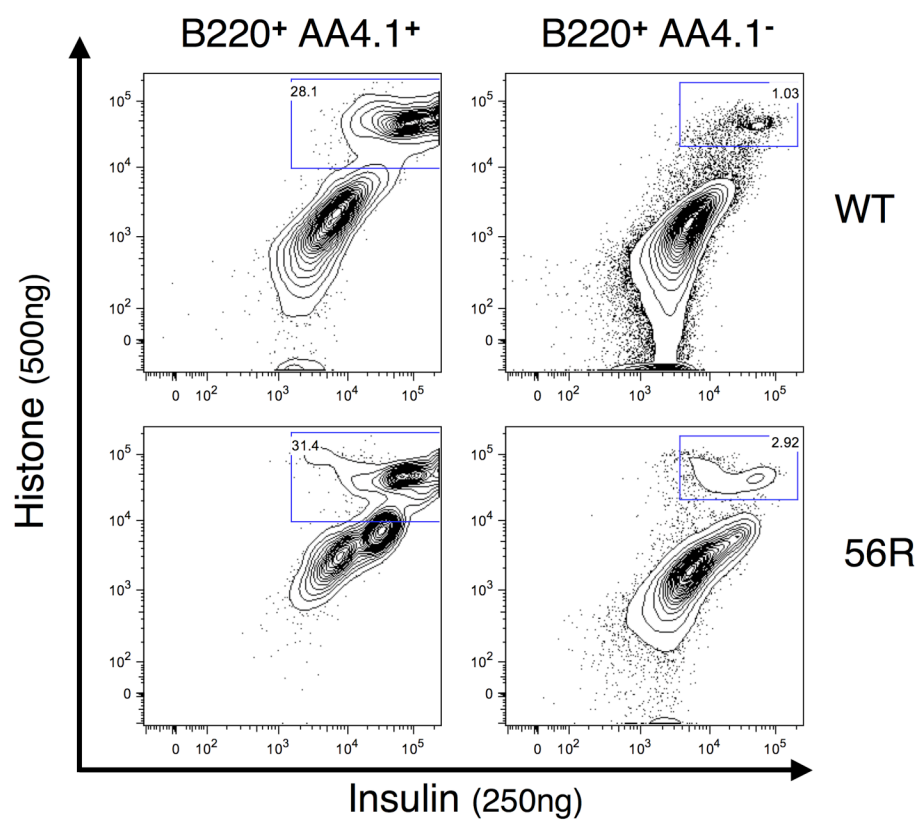
**Figure 4-1: Bcl-x<sub>L</sub> expression increases Vκ-RS rearrangement levels in immature bone marrow B cells.** Vκ-RS rearrangement levels in genomic DNA from bone marrow (Fr. D, Fr. E Igκ<sup>+</sup>, and Fr. F Igκ<sup>+</sup>) B cells of Bcl-x<sub>L</sub> transgenic (n=3, gray bars). Data from C57Bl/6 mice (n=5, black bars) are plotted for comparison. All mice were 2-3 months of age. All PCRs were performed in duplicate. Data represent fold difference relative to the mean RS level in C57Bl/6 B220<sup>+</sup> IgM<sup>+</sup> Igκ<sup>+</sup> splenic B cells (+s.e.m., \* = p<0.05; one-tailed t-test).

**Figure 4-2**

**a**



**b**



**Figure 4-2: Auto-antigen staining of C57Bl/6 and 56R<sup>+/-</sup> splenic B cells.** (a) Live (7-AAD<sup>-</sup>) B220<sup>+</sup> splenic lymphocytes from wild-type (left) and 56R<sup>+/-</sup> (middle) mice stained with 500ng insulin (total volume 100μL; pre-conjugated to SA<sub>v</sub>-FITC in a 4:1 ratio). The histogram plot on the right depicts insulin staining of wild-type (red) and 56R<sup>+/-</sup> (blue) splenic marginal zone B cells (CD21<sup>+</sup> CD23<sup>-</sup>). (b) Live (7-AAD<sup>-</sup>) immature (B220<sup>+</sup> AA4.1<sup>+</sup>, left) and mature (B220<sup>+</sup> AA4.1<sup>-</sup>, right) splenic lymphocytes from wild-type (top) and 56R<sup>+/-</sup> (bottom) mice stained with 250ng insulin (pre-conjugated to SA<sub>v</sub>-FITC in a 4:1 ratio) and 500ng histone H1 conjugated to PerCP (total volume 100μL).

## **CHAPTER 5: Materials and Methods**

## **Mice**

B6.56R mice have been described previously (Sekiguchi et al. 2006). C57Bl/6, NOD, NOR, MRL/lpr, and MRL/MpJ mice were obtained from Jackson Laboratories. AEC mice were originally a gift from Philip Cohen. Bcl-x<sub>L</sub> mice have been previously described (Gonzalez-Garcia et al. 1994). All animal experiments were performed on 3–4 month old mice in accordance with protocols approved by the University of Pennsylvania School of Medicine Animal Care and Use Committee.

## **Flow Cytometry**

Murine cell suspensions were prepared from femurs, tibias, and spleens in FACS buffer (PBS, 0.5% BSA, 0.01% NaN<sub>3</sub>, 1mM EDTA) following hypotonic RBC lysis (ACK Lysing Buffer, BioWhittaker). B cell populations were defined as described in Figure 2–2. Due to poor reactivity of AA4.1 antibodies in NOD and NOR mice (Langmuir et al. 1993), bone marrow and peripheral subsets in these mice were defined using a method that has been described previously by others (Figure 2–2, (Quinn et al. 2006)). Human B cells were isolated from whole blood using Lymphocyte Separation Medium (MP Biomedicals, Solon, OH) followed by resuspension in FACS buffer. Murine lymphocytes were stained using anti-IgM-PE-Cy7 (II/41), anti-IgD-PE (11–26), anti-B220-APC-AF750 (RA3–6B2) (eBioscience), and anti-Igκ-FITC (187.1), anti-BP-1-FITC (6C3), anti-CD43-PE (S7), anti-CD23-PE (B3B4), anti-AA4.1-APC, (BD-Pharmigen). Human lymphocytes were stained with anti-CD19-Pacific Blue (Caltag Laboratories) and anti-Igλ-FITC, anti-CD27-FITC, anti-Igκ-PE, anti-CD38-PE-Cy7 (BD Pharmingen). To maximize efficient use of patient samples for additional experiments not described here, CD19<sup>+</sup> B cells marked by the anti-idiotypic monoclonal antibody 9G4, which recognizes V<sub>H</sub>4–34 heavy chain rearrangements (Isenberg et al. 1993), were excluded from human CD19<sup>+</sup> B cell populations described herein (Figure 2–2d). 9G4Id<sup>+</sup> cells

comprised 5.1%, 5.7%, and 4.2% of total CD19<sup>+</sup> B cells from control, SLE, and T1D subjects, respectively. All cells were sorted using the BD FACSaria with sort purities over 90%.

### **Quantitative PCR**

Genomic DNA was isolated from sorted B cells using the Gentra PureGene Tissue Kit (Qiagen). Quantitative PCR (40°C 10 min., 95°C 10min., followed by 60 cycles at 95°C 10 sec., 60°C 30 sec., 72°C 1 sec.) was performed on 15–50ng template DNA in a 20μL reaction mix containing 1X LightCycler 480 Probes Master Mix (Roche Applied Science), 0.5U LightCycler Uracil-DNA Glycosylase (Roche Applied Science), 0.5μM forward primer, 0.5μM reverse primer, and 0.2μM hydrolysis probe using a LightCycler 480 real-time PCR system (Roche Applied Science). V<sub>κ</sub>-RS rearrangements were amplified with 5'-GGCTGCAGSTTCAGTGGCAGTGGRTCWGGAC-3' (Schlissel and Baltimore 1989) and 5'-CTGAGCTCAACTGCAGTCCTA-3' primers and detected with 5'-TGGCAGCCCAGGGTGGATCT-3' FAM-labeled hydrolysis probe. For each sample, the intronic region of the reference control gene β-actin was amplified in a separate well with forward primer 5'-GAGGCTCTTTTCCAGCCTTC-3' and reverse primer 5'-TCCATACCTAAGAGAAGAGTGACAGA-3' and detected with a Cy5-labeled hydrolysis probe 5'-AAGGTGACAAAACCTCCTGAGGCCA-3'. iRS-KDE rearrangements were amplified with 5'-ATTGATGCTGCCGTAGCC-3' and 5'-AGGCTTCCTAGGGAGGTCAG-3' primers and detected with 5'-TCTGCAGCTGCATTTTGGCA-3' FAM-labeled hydrolysis probe. Similarly, for each sample, the intronic region of the reference control gene β-actin was amplified in a separate well with forward primer 5'-CCCAGCACAAATGAAGATCAA-3' and reverse primer 5'-AGTACTTGCGCTCAGGAGGA-3' and detected with a Cy5-labeled hydrolysis probe 5'-TGCCTGAGCTGACCTGGGCA-3'. For V<sub>κ</sub>-RS quantitation each product (V<sub>κ</sub>-RS or β-actin) was analyzed by the standard curve method (titrated cloned V<sub>κ</sub>-RS or NIH3T3 genomic

DNA, respectively) and the amount of V $\kappa$ -RS product in each mouse sample was then normalized to the amount of  $\beta$ -actin product. Samples were compared to the normalized target value in wild-type C57Bl/6 B220<sup>+</sup> IgM<sup>+</sup>  $\kappa$ <sup>+</sup> splenocytes to determine a relative quantity (comparative C<sub>T</sub> (DDC<sub>T</sub>) method). The frequency of iRS-KDE rearrangements in each human sample was determined via absolute quantification using a standard curve generated by serial dilution of a cloned iRS-KDE rearrangement resuspended with 100ng human fibroblast DNA (Fig. 2–3). To determine the number of genome copies,  $\beta$ -actin was measured via absolute quantification using a standard curve consisting of serially diluted human fibroblast DNA. Reactions were performed in duplicate and samples with inconsistent replicates or  $\beta$ -actin cycle numbers greater than 35 were excluded.

#### V $\kappa$ -RS and iRS-KDE Standard Curve

To generate standard curves for V $\kappa$ -RS and iRS-KDE quantitative PCR, V $\kappa$ -RS and iRS-KDE rearrangements were cloned from murine and human lymphocytes, respectively. The cloned V $\kappa$ -RS rearrangement sequence is as follows (primers shown in bold):

5' **GGCTGCAGGTTTCAGTGGCAGTGGATCAGGGTC**AGATTTCACTCTCAGTATCAACAGTGTGGAACCTGAA  
GATTTTGGAAATGTATTTCTGTCAACAGAGTAACAGCTGGCCTGACCCTAGTGGCAGCCCAGGGTGGATCTC  
CCT**AGGACTGCAGTTGAGCTCAG** 3'

The cloned iRS-KDE rearrangement sequence is as follows (primers shown in bold):

5' **ATTGATGCTGCCGTAGCC**AGCTTTCTGTTGAGTGGCAGCCCAGGGCGACTCCTCATGAGTCTGCAGCT  
GCATTTTTGCCATATCCACTATTTGGAGT**CTGACCTCCCTAGGAAGCCT** 3'

Each plasmid was serially diluted 10-fold into Tris-EDTA buffer eight times beginning with concentrations of 2.5ng/ $\mu$ L. V $\kappa$ -RS and iRS-KDE serial dilutions were

combined with NIH3T3 (mouse) and LM216 (human) fibroblast DNA, respectively, yielding final fibroblast DNA concentrations of 20ng/ $\mu$ L. The number of RS rearrangement copies in each dilution was quantified using the respective plasmid molecular weight ( $V_{\kappa}$ -RS = 2609777 g/mol; iRS-KDE = 2583122 g/mol).

### **Human Subjects**

Volunteer healthy adult subjects with no history of autoimmune disease, no active viral or bacterial infection, or current use of immunomodulatory or immunosuppressive drugs were recruited. Women who were nursing, pregnant, or who were planning on becoming pregnant during the duration of this study were excluded. Adults with documented SLE (fulfilling the ARA criteria (Tan et al. 1982)) were recruited from the Rheumatology clinic at the Hospital of the University of Pennsylvania. Blood draws from SLE patients receiving steroids were performed prior to their first morning dose. T1D patients were recruited from the Rodebaugh Diabetes Center at the Hospital of the University of Pennsylvania. Subjects were included if they had a clinical history compatible with autoimmune T1D, defined by insulin-dependence, the absence of obesity, and an age of onset < 40 years or > 40 years together with elevated levels of autoantibodies to GAD65. Subjects meeting these criteria but presently receiving immunosuppression drugs to support a kidney, pancreas, or islet cell transplant were excluded. The study protocol was approved by the Institutional Review Board of the University of Pennsylvania and all subjects gave their written informed consent to participate.

### **RS Precursor Isolation**

RS editing precursors were PCR amplified (95°C 10min., followed by 45 cycles at 95°C 10 sec., 60°C 30 sec., 72°C 30 sec.) using forward  $V_{\kappa}$  gene specific primers ( $V_{\kappa}$ 12:



5'-CGAGCAAGTGAGAATATTTACAGTAATTTAGC-3'; V $\kappa$ 38c: 5'-CTCATAACATTACACATCTACAT TACAGCC-3') or a forward degenerate V $\kappa$  primer (5'-GGCTGCAGSTTCAGTGGCAGTGGR TCWGGGRAC-3') and an RS specific reverse primer (5'-CTGAGCTCAACTGCAGTCCTA-3'). Amplifications were performed on 10ng total spleen genomic DNA in a 20 $\mu$ L reaction mix containing 1.25U Failsafe Taq Polymerase PCR Mix (Epicentre Biotechnologies), 1X Failsafe PCR Buffer D (Epicentre Biotechnologies), 1 $\mu$ M forward primer, 1 $\mu$ M reverse primer. PCR products were visualized by agarose gel (1.0%) electrophoresis. Target products of 1.5kbp were isolated using the QIAquick gel extraction kit (Qiagen) and cloned using the TA TOPO Cloning Kit (Invitrogen).

### Insulin Staining of Fresh Splenocytes for Flow Cytometric Analysis

Insulin – FW: 5733.49 g/mol	Insulin-biotin – Sigma #I2258
Biotin – FW: 244.31 g/mol	
Streptavidin – 13kDa = FW: 51677.34 g/mol	
FITC – FW: 389.38 g/mol	FITC-streptavidin – BD Pharmingen
#554060	

### Pre-conjugate insulin-bio and FITC-sAV in FACS Buffer on ice.

FACS Buffer: 1L PBS + 0.5% BSA + 0.01% NaN<sub>3</sub> (1mL 10%NaN<sub>3</sub>)

#### *Cell Staining*

1. Centrifuge cells at 1300rpm for 7 minutes at 4°C.
2. Aspirate as much media as possible and add 250 $\mu$ L FACS buffer (when < 50x10<sup>6</sup> cells) or 350 $\mu$ L FACS buffer (when ~ 100x10<sup>6</sup> cells) (i.e. enough FACS to allow for easy transfer of 1x10<sup>6</sup> cells to staining tubes).
3. Transfer 1x10<sup>6</sup> cells into each compensation tube and 1x10<sup>6</sup> cells to each analysis tube.
4. Add 2mL FACS buffer to each tube. Centrifuge at 1300rpm for 5 minutes at 4°C.
5. Aspirate supernatant and vortex tubes to break up the cell pellet. Add Fc-block, vortex, and incubate on ice for 5 minutes.
6. Add **staining antibody mix** and vortex to distribute mix.
7. **Combine insulin-biotin and SA-FITC in 100 $\mu$ L FACS buffer in ratios listed below.**
8. Incubate tubes in the dark on ice for 20 minutes.
9. Add 2mL FACS buffer to the tubes to wash cells. Centrifuge tubes at 1300rpm for 5 minutes at 4°C. Aspirate supernatant and vortex tubes to resuspend cells.
10. Repeat wash as described in step 7.
11. Aspirate supernatant and vortex tubes to break up the cell pellet. Add 100 $\mu$ L to compensation tubes and add **streptavidin staining mix or preconjugated insulin** to analysis. Vortex briefly to distribute mix.

12. Incubate tubes in the dark on ice for 20 minutes.
13. Add 2mL FACS buffer to the tubes to wash cells. Centrifuge tubes at 1300rpm for 5 minutes at 4°C. Aspirate supernatant and vortex tubes to resuspend cells.
14. Repeat wash **twice** as described in step 11.
15. Resuspend cells in 0.4ml FACS buffer.

**Insulin-biotin + SA-FITC (4:1) Preconjugated**

Tube	Sample	FITC	PE	7-AAD	PE-Cy7	APC
<b>#1 Spleen 1000ng</b>	Splenocytes	Insulin (1000ng) $1.67 \times 10^{-10}$ mol insulin (5 $\mu$ L) $4.175 \times 10^{-11}$ mol SA-FITC (4.355 $\mu$ L)	CD23	7-AAD	CD45R (B220)	CD21
<b>#2 Spleen 500ng</b>	Splenocytes	Insulin (500ng) $8.35 \times 10^{-11}$ mol insulin (2.5 $\mu$ L) $2.09 \times 10^{-11}$ mol SA-FITC (2.178 $\mu$ L)	CD23	7-AAD	CD45R (B220)	CD21
<b>#3 Spleen 250ng</b>	Splenocytes	Insulin (250ng) $4.175 \times 10^{-11}$ mol insulin (1.25 $\mu$ L) $1.044 \times 10^{-11}$ mol SA-FITC (1.089 $\mu$ L)	CD23	7-AAD	CD45R (B220)	CD21
<b>#4 Spleen 100ng</b>	Splenocytes	Insulin (100ng) $1.67 \times 10^{-11}$ mol insulin (0.5 $\mu$ L) $4.175 \times 10^{-12}$ mol SA-FITC (0.436 $\mu$ L)	CD23	7-AAD	CD45R (B220)	CD21
<b>#5 Spleen 50ng</b>	Splenocytes	Insulin (50ng) $8.35 \times 10^{-12}$ mol insulin (0.25 $\mu$ L) $2.09 \times 10^{-12}$ mol SA-FITC (0.218 $\mu$ L)	CD23	7-AAD	CD45R (B220)	CD21

**Statistical Analysis**

Group comparisons for the mouse experiments were performed with a two-tailed Student's t-test unless otherwise specified. Group comparisons in human subjects were performed via one-way ANOVA followed by two-tailed Student's t-test. Correlation analyses were performed by calculating Spearman's rank correlation coefficients. Categorical data (gender, race) comparisons were analyzed using Fisher's exact test. For all tests a p value of  $\leq 0.05$  was considered significant.

## REFERENCES

- Ahuja, A., J. Shupe, et al. (2007). "Depletion of B cells in murine lupus: efficacy and resistance." J Immunol **179**(5): 3351–61.
- Aiba, Y., M. Kameyama, et al. (2008). "Regulation of B-cell development by BCAP and CD19 through their binding to phosphoinositide 3-kinase." Blood **111**(3): 1497–503.
- Allman, D., R. C. Lindsley, et al. (2001). "Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation." J Immunol **167**(12): 6834–40.
- Alt, F. W., G. D. Yancopoulos, et al. (1984). "Ordered rearrangement of immunoglobulin heavy chain variable region segments." Embo J **3**(6): 1209–19.
- Amin, R. H. and M. S. Schlissel (2008). "Foxo1 directly regulates the transcription of recombination-activating genes during B cell development." Nat Immunol **9**(6): 613–22.
- Arakawa, H., T. Shimizu, et al. (1996). "Re-evaluation of the probabilities for productive arrangements on the kappa and lambda loci." Int Immunol **8**(1): 91–9.
- Bai, L., Y. Chen, et al. (2007). "Phospholipase Cgamma2 contributes to light-chain gene activation and receptor editing." Mol Cell Biol **27**(17): 5957–67.
- Benschop, R. J., K. Aviszus, et al. (2001). "Activation and anergy in bone marrow B cells of a novel immunoglobulin transgenic mouse that is both hapten specific and autoreactive." Immunity **14**(1): 33–43.
- Biggs, W. H., 3rd, J. Meisenholder, et al. (1999). "Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1." Proc Natl Acad Sci U S A **96**(13): 7421–6.
- Blunt, T., N. J. Finnie, et al. (1995). "Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation." Cell **80**(5): 813–23.
- Bottini, N., L. Musumeci, et al. (2004). "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes." Nat Genet **36**(4): 337–8.
- Bradl, H. and H. M. Jack (2001). "Surrogate light chain-mediated interaction of a soluble pre-B cell receptor with adherent cell lines." J Immunol **167**(11): 6403–11.
- Braun, U., K. Rajewsky, et al. (2000). "Different sensitivity to receptor editing of B cells from mice hemizygous or homozygous for targeted Ig transgenes." Proc Natl Acad Sci U S A **97**(13): 7429–34.
- Brauninger, A., T. Goossens, et al. (2001). "Regulation of immunoglobulin light chain gene rearrangements during early B cell development in the human." Eur J Immunol **31**(12): 3631–7.

- Brayer, J., J. Lowry, et al. (2000). "Alleles from chromosomes 1 and 3 of NOD mice combine to influence Sjogren's syndrome-like autoimmune exocrinopathy." J Rheumatol **27**(8): 1896-904.
- Brown, K. E., S. S. Guest, et al. (1997). "Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin." Cell **91**(6): 845-54.
- Brown, S. T., G. A. Miranda, et al. (1997). "Regulation of the RAG-1 promoter by the NF- $\kappa$ B transcription factor." J Immunol **158**(11): 5071-4.
- Cariappa, A., C. Chase, et al. (2007). "Naive recirculating B cells mature simultaneously in the spleen and bone marrow." Blood **109**(6): 2339-45.
- Casellas, R., T. A. Shih, et al. (2001). "Contribution of receptor editing to the antibody repertoire." Science **291**(5508): 1541-4.
- Casellas, R., Q. Zhang, et al. (2007). "Igkappa allelic inclusion is a consequence of receptor editing." J Exp Med **204**(1): 153-60.
- Cha, S., H. Nagashima, et al. (2002). "Two NOD Idd-associated intervals contribute synergistically to the development of autoimmune exocrinopathy (Sjogren's syndrome) on a healthy murine background." Arthritis Rheum **46**(5): 1390-8.
- Cheema, G. S., V. Roschke, et al. (2001). "Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases." Arthritis Rheum **44**(6): 1313-9.
- Chen, C., Z. Nagy, et al. (1995). "Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing." Immunity **3**(6): 747-55.
- Chen, C., E. L. Prak, et al. (1997). "Editing disease-associated autoantibodies." Immunity **6**(1): 97-105.
- Chen, J., M. Trunstin, et al. (1993). "B cell development in mice that lack one or both immunoglobulin kappa light chain genes." Embo J **12**(3): 821-30.
- Chen, Q., W. Yang, et al. (2008). "IRF-4-binding protein inhibits interleukin-17 and interleukin-21 production by controlling the activity of IRF-4 transcription factor." Immunity **29**(6): 899-911.
- Cheng, A. M., B. Rowley, et al. (1995). "Syk tyrosine kinase required for mouse viability and B-cell development." Nature **378**(6554): 303-6.
- Chowdhury, D. and R. Sen (2001). "Stepwise activation of the immunoglobulin mu heavy chain gene locus." Embo J **20**(22): 6394-403.
- Cloutier, J. F. and A. Veillette (1999). "Cooperative inhibition of T-cell antigen receptor signaling by a complex between a kinase and a phosphatase." J Exp Med **189**(1): 111-21.
- Coffer, P. J. and B. M. Burgering (2004). "Forkhead-box transcription factors and their role in the immune system." Nat Rev Immunol **4**(11): 889-99.

- Cohen, S., H. Dadi, et al. (1999). "Cloning and characterization of a lymphoid-specific, inducible human protein tyrosine phosphatase, Lyp." Blood **93**(6): 2013–24.
- Cohn, M. and R. E. Langman (1990). "The protection: the unit of humoral immunity selected by evolution." Immunol Rev **115**: 11–147.
- Coleclough, C., R. P. Perry, et al. (1981). "Aberrant rearrangements contribute significantly to the allelic exclusion of immunoglobulin gene expression." Nature **290**(5805): 372–8.
- Cooke, M. P., A. W. Heath, et al. (1994). "Immunoglobulin signal transduction guides the specificity of B cell–T cell interactions and is blocked in tolerant self-reactive B cells." J Exp Med **179**(2): 425–38.
- Cornall, R. J., J. G. Cyster, et al. (1998). "Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection." Immunity **8**(4): 497–508.
- Crouzier, R., T. Martin, et al. (1995). "Heavy chain variable region, light chain variable region, and heavy chain CDR3 influences on the mono- and polyreactivity and on the affinity of human monoclonal rheumatoid factors." J Immunol **154**(9): 4526–35.
- Daitch, L. E., M. W. Moore, et al. (1992). "Transcription and recombination of the murine RS element." J Immunol **149**(3): 832–40.
- Dammers, P. M., A. Visser, et al. (2000). "Most marginal zone B cells in rat express germline encoded Ig VH genes and are ligand selected." J Immunol **165**(11): 6156–69.
- Dorshkind, K. and E. Montecino-Rodriguez (2007). "Fetal B-cell lymphopoiesis and the emergence of B-1-cell potential." Nat Rev Immunol **7**(3): 213–9.
- Doyle, C. M., J. Han, et al. (2006). "Consequences of receptor editing at the lambda locus: multireactivity and light chain secretion." Proc Natl Acad Sci U S A **103**(30): 11264–9.
- Dunda, O. and D. Corcos (1997). "Recombining sequence recombination in normal kappa-chain-expressing B cells." J Immunol **159**(9): 4362–6.
- Durdik, J., M. W. Moore, et al. (1984). "Novel kappa light-chain gene rearrangements in mouse lambda light chain-producing B lymphocytes." Nature **307**(5953): 749–52.
- Eisenbeis, C. F., H. Singh, et al. (1995). "Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator." Genes Dev **9**(11): 1377–87.
- Eisenberg, R. (2006). "Targeting B cells in SLE: the experience with rituximab treatment (anti-CD20)." Endocr Metab Immune Disord Drug Targets **6**(4): 345–50.
- Engler, P., D. Haasch, et al. (1991). "A strain-specific modifier on mouse chromosome 4 controls the methylation of independent transgene loci." Cell **65**(6): 939–47.

- Erikson, J., M. Z. Radic, et al. (1991). "Expression of anti-DNA immunoglobulin transgenes in non-autoimmune mice." Nature **349**(6307): 331-4.
- Florez, J. C., J. Hirschhorn, et al. (2003). "The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits." Annu Rev Genomics Hum Genet **4**: 257-91.
- Fuentes-Panana, E. M., G. Bannish, et al. (2004). "Basal Igalpha/Igbeta signals trigger the coordinated initiation of pre-B cell antigen receptor-dependent processes." J Immunol **173**(2): 1000-11.
- Fulcher, D. A., A. B. Lyons, et al. (1996). "The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help." J Exp Med **183**(5): 2313-28.
- Fuller, K. and U. Storb (1997). "Identification and characterization of the murine Rag1 promoter." Mol Immunol **34**(12-13): 939-54.
- Gauthier, L., B. Rossi, et al. (2002). "Galectin-1 is a stromal cell ligand of the pre-B cell receptor (BCR) implicated in synapse formation between pre-B and stromal cells and in pre-BCR triggering." Proc Natl Acad Sci U S A **99**(20): 13014-9.
- Gay, D., T. Saunders, et al. (1993). "Receptor editing: an approach by autoreactive B cells to escape tolerance." J Exp Med **177**(4): 999-1008.
- Glinksky, G. V. (2008). "An SNP-guided microRNA map of fifteen common human disorders identifies a consensus disease phenocode aiming at principal components of the nuclear import pathway." Cell Cycle **7**(16).
- Golding, A., S. Chandler, et al. (1999). "Nucleosome structure completely inhibits in vitro cleavage by the V(D)J recombinase." Embo J **18**(13): 3712-23.
- Gong, S. and M. C. Nussenzweig (1996). "Regulation of an early developmental checkpoint in the B cell pathway by Ig beta." Science **272**(5260): 411-4.
- Gonzalez-Garcia, M., R. Perez-Ballester, et al. (1994). "bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria." Development **120**(10): 3033-42.
- Goodnow, C. C., J. Crosbie, et al. (1988). "Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice." Nature **334**(6184): 676-82.
- Gorman, J. R., N. van der Stoep, et al. (1996). "The Ig(kappa) enhancer influences the ratio of Ig(kappa) versus Ig(lambda) B lymphocytes." Immunity **5**(3): 241-52.
- Grawunder, U., T. M. Leu, et al. (1995). "Down-regulation of RAG1 and RAG2 gene expression in preB cells after functional immunoglobulin heavy chain rearrangement." Immunity **3**(5): 601-8.
- Gross, A. J., J. R. Lyandres, et al. (2009). "Developmental Acquisition of the Lyn-CD22-SHP-1 Inhibitory Pathway Promotes B Cell Tolerance." J Immunol **182**(9).

- Guo, B., R. M. Kato, et al. (2000). "Engagement of the human pre-B cell receptor generates a lipid raft-dependent calcium signaling complex." *Immunity* **13**(2): 243–53.
- Guo, J., A. Hawwari, et al. (2002). "Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes." *Nat Immunol* **3**(5): 469–76.
- Halverson, R., R. M. Torres, et al. (2004). "Receptor editing is the main mechanism of B cell tolerance toward membrane antigens." *Nat Immunol* **5**(6): 645–50.
- Harada, K. and H. Yamagishi (1991). "Lack of feedback inhibition of V kappa gene rearrangement by productively rearranged alleles." *J Exp Med* **173**(2): 409–15.
- Hardy, R. R., C. E. Carmack, et al. (1991). "Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow." *J Exp Med* **173**(5): 1213–25.
- Hardy, R. R., P. W. Kincade, et al. (2007). "The protean nature of cells in the B lymphocyte lineage." *Immunity* **26**(6): 703–14.
- Harley, J. B., M. E. Alarcon-Riquelme, et al. (2008). "Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PTK, KIAA1542 and other loci." *Nat Genet* **40**(2): 204–10.
- Hartley, S. B., M. P. Cooke, et al. (1993). "Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death." *Cell* **72**(3): 325–35.
- Hartley, S. B., J. Crosbie, et al. (1991). "Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens." *Nature* **353**(6346): 765–9.
- Hayakawa, K., M. Asano, et al. (1999). "Positive selection of natural autoreactive B cells." *Science* **285**(5424): 113–6.
- Hayakawa, K., M. Asano, et al. (2003). "Positive selection of anti-thy-1 autoreactive B-1 cells and natural serum autoantibody production independent from bone marrow B cell development." *J Exp Med* **197**(1): 87–99.
- Hayakawa, K., R. R. Hardy, et al. (1985). "Progenitors for Ly-1 B cells are distinct from progenitors for other B cells." *J Exp Med* **161**(6): 1554–68.
- Hayakawa, K., R. R. Hardy, et al. (1984). "Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies." *Proc Natl Acad Sci U S A* **81**(8): 2494–8.
- Hayashi, K., T. Nojima, et al. (2004). "Impaired receptor editing in the primary B cell repertoire of BASH-deficient mice." *J Immunol* **173**(10): 5980–8.
- Hayashi, K., M. Yamamoto, et al. (2003). "Distinct signaling requirements for Dmu selection, IgH allelic exclusion, pre-B cell transition, and tumor suppression in B cell progenitors." *Immunity* **18**(6): 825–36.

- Hertz, M. and D. Nemazee (1997). "BCR ligation induces receptor editing in IgM+IgD-bone marrow B cells in vitro." Immunity **6**(4): 429–36.
- Herzog, S., E. Hug, et al. (2008). "SLP-65 regulates immunoglobulin light chain gene recombination through the PI(3)K-PKB-Foxo pathway." Nat Immunol **9**(6): 623–31.
- Herzog, S., M. Reth, et al. (2009). "Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling." Nat Rev Immunol **9**(3): 195–205.
- Hesslein, D. G., D. L. Pflugh, et al. (2003). "Pax5 is required for recombination of transcribed, acetylated, 5' IgH V gene segments." Genes Dev **17**(1): 37–42.
- Hewicker, M., E. Kromschroder, et al. (1990). "Detection of circulating immune complexes in MRL mice with different forms of glomerulonephritis." Z Versuchstierkd **33**(4): 149–56.
- Hibbs, M. L., D. M. Tarlinton, et al. (1995). "Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease." Cell **83**(2): 301–11.
- Hippen, K. L., B. R. Schram, et al. (2005). "In vivo assessment of the relative contributions of deletion, anergy, and editing to B cell self-tolerance." J Immunol **175**(2): 909–16.
- Holman, P. O., E. R. Walsh, et al. (2003). "The central tolerance response to male antigen in normal mice is deletion and not receptor editing." J Immunol **171**(8): 4048–53.
- Hsieh, C. L. and M. R. Lieber (1992). "CpG methylated minichromosomes become inaccessible for V(D)J recombination after undergoing replication." Embo J **11**(1): 315–25.
- Hsu, L. Y., J. Lanning, et al. (2003). "A conserved transcriptional enhancer regulates RAG gene expression in developing B cells." Immunity **19**(1): 105–17.
- Hu, C. Y., D. Rodriguez-Pinto, et al. (2007). "Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice." J Clin Invest **117**(12): 3857–67.
- Ichiyoshi, Y. and P. Casali (1994). "Analysis of the structural correlates for antibody polyreactivity by multiple reassortments of chimeric human immunoglobulin heavy and light chain V segments." J Exp Med **180**(3): 885–95.
- Igarashi, H., S. C. Gregory, et al. (2002). "Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow." Immunity **17**(2): 117–30.
- Inlay, M., F. W. Alt, et al. (2002). "Essential roles of the kappa light chain intronic enhancer and 3' enhancer in kappa rearrangement and demethylation." Nat Immunol **3**(5): 463–8.
- Inlay, M. A., H. Tian, et al. (2004). "Important roles for E protein binding sites within the immunoglobulin kappa chain intronic enhancer in activating V kappa J kappa rearrangement." J Exp Med **200**(9): 1205–11.



- Isenberg, D., M. Spellerberg, et al. (1993). "Identification of the 9G4 idiotope in systemic lupus erythematosus." Br J Rheumatol **32**(10): 876–82.
- Jameson, S. C., K. A. Hogquist, et al. (1995). "Positive selection of thymocytes." Annu Rev Immunol **13**: 93–126.
- Janeway, C., K. P. Murphy, et al. (2008). Janeway's immunobiology. New York, NY, Garland Science.
- Jin, Z. X., H. Kishi, et al. (2002). "Lymphoid enhancer-binding factor-1 binds and activates the recombination-activating gene-2 promoter together with c-Myb and Pax-5 in immature B cells." J Immunol **169**(7): 3783–92.
- Johnson, K., T. Hashimshony, et al. (2008). "Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling." Immunity **28**(3): 335–45.
- Kanno, H., M. Nose, et al. (1992). "Spontaneous development of pancreatitis in the MRL/Mp strain of mice in autoimmune mechanism." Clin Exp Immunol **89**(1): 68–73.
- Khan, S. N., E. J. Witsch, et al. (2008). "Editing and escape from editing in anti-DNA B cells." Proc Natl Acad Sci U S A **105**(10): 3861–6.
- Kirschbaum, T., S. Pourrajabi, et al. (1998). "The 3' part of the immunoglobulin kappa locus of the mouse." Eur J Immunol **28**(5): 1458–66.
- Kirschbaum, T., F. Roschenthaler, et al. (1999). "The central part of the mouse immunoglobulin kappa locus." Eur J Immunol **29**(7): 2057–64.
- Kishi, H., Z. X. Jin, et al. (2002). "Cooperative binding of c-Myb and Pax-5 activates the RAG-2 promoter in immature B cells." Blood **99**(2): 576–83.
- Kishimoto, H. and J. Sprent (2001). "A defect in central tolerance in NOD mice." Nat Immunol **2**(11): 1025–31.
- Kitamura, D., A. Kudo, et al. (1992). "A critical role of lambda 5 protein in B cell development." Cell **69**(5): 823–31.
- Kitamura, D., J. Roes, et al. (1991). "A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene." Nature **350**(6317): 423–6.
- Klein, F., N. Feldhahn, et al. (2005). "Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary IGK gene rearrangement, IGK deletion, and IGL gene rearrangement." J Immunol **174**(1): 367–75.
- Klonowski, K. D., L. L. Primiano, et al. (1999). "Atypical VH-D-JH rearrangements in newborn autoimmune MRL mice." J Immunol **162**(3): 1566–72.

- Kohler, F., E. Hug, et al. (2008). "Autoreactive B cell receptors mimic autonomous pre-B cell receptor signaling and induce proliferation of early B cells." *Immunity* **29**(6): 912–21.
- Koretzky, G. A., F. Abtahian, et al. (2006). "SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond." *Nat Rev Immunol* **6**(1): 67–78.
- Kosak, S. T., J. A. Skok, et al. (2002). "Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development." *Science* **296**(5565): 158–62.
- Kraus, M., M. B. Alimzhanov, et al. (2004). "Survival of resting mature B lymphocytes depends on BCR signaling via the Igalpha/beta heterodimer." *Cell* **117**(6): 787–800.
- Kwon, J., A. N. Imbalzano, et al. (1998). "Accessibility of nucleosomal DNA to V(D)J cleavage is modulated by RSS positioning and HMG1." *Mol Cell* **2**(6): 829–39.
- Kwon, J., K. B. Morshead, et al. (2000). "Histone acetylation and hSWI/SNF remodeling act in concert to stimulate V(D)J cleavage of nucleosomal DNA." *Mol Cell* **6**(5): 1037–48.
- Kyogoku, C., C. D. Langefeld, et al. (2004). "Genetic association of the R620W polymorphism of protein tyrosine phosphatase PTPN22 with human SLE." *Am J Hum Genet* **75**(3): 504–7.
- Lam, K. P., R. Kuhn, et al. (1997). "In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death." *Cell* **90**(6): 1073–83.
- Lamoureux, J. L., L. C. Watson, et al. (2007). "Reduced receptor editing in lupus-prone MRL/lpr mice." *J Exp Med* **204**(12): 2853–64.
- Langmuir, P. B., M. M. Bridgett, et al. (1993). "Bone marrow abnormalities in the non-obese diabetic mouse." *Int Immunol* **5**(2): 169–77.
- Lauring, J. and M. S. Schlissel (1999). "Distinct factors regulate the murine RAG-2 promoter in B- and T-cell lines." *Mol Cell Biol* **19**(4): 2601–12.
- Lazorchak, A. S., M. S. Schlissel, et al. (2006). "E2A and IRF-4/Pip promote chromatin modification and transcription of the immunoglobulin kappa locus in pre-B cells." *Mol Cell Biol* **26**(3): 810–21.
- Leandro, M. J., J. C. Edwards, et al. (2002). "An open study of B lymphocyte depletion in systemic lupus erythematosus." *Arthritis Rheum* **46**(10): 2673–7.
- Lee, Y. H., Y. H. Rho, et al. (2007). "The PTPN22 C1858T functional polymorphism and autoimmune diseases--a meta-analysis." *Rheumatology (Oxford)* **46**(1): 49–56.
- Lesley, R., Y. Xu, et al. (2004). "Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF." *Immunity* **20**(4): 441–53.

- Leslie, R. D. and M. Delli Castelli (2004). "Age-dependent influences on the origins of autoimmune diabetes: evidence and implications." Diabetes **53**(12): 3033–40.
- Lewis, S., N. Rosenberg, et al. (1982). "Continuing kappa-gene rearrangement in a cell line transformed by Abelson murine leukemia virus." Cell **30**(3): 807–16.
- Li, H., Y. Jiang, et al. (2003). "Regulation of anti-phosphatidylserine antibodies." Immunity **18**(2): 185–92.
- Li, H., Y. Jiang, et al. (2001). "Editors and editing of anti-DNA receptors." Immunity **15**(6): 947–57.
- Li, Y., H. Li, et al. (2002). "Autoreactive B cells in the marginal zone that express dual receptors." J Exp Med **195**(2): 181–8.
- Li, Y. S., R. Wasserman, et al. (1996). "Identification of the earliest B lineage stage in mouse bone marrow." Immunity **5**(6): 527–35.
- Lin, L., J. D. Hron, et al. (2004). "Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a." Immunity **21**(2): 203–13.
- Lindsley, R. C., M. Thomas, et al. (2007). "Generation of peripheral B cells occurs via two spatially and temporally distinct pathways." Blood **109**(6): 2521–8.
- Liu, S., M. G. Velez, et al. (2005). "Receptor editing can lead to allelic inclusion and development of B cells that retain antibodies reacting with high avidity autoantigens." J Immunol **175**(8): 5067–76.
- Liu, Y., L. Li, et al. (2007). "Lupus susceptibility genes may breach tolerance to DNA by impairing receptor editing of nuclear antigen-reactive B cells." J Immunol **179**(2): 1340–52.
- Liu, Y., L. Li, et al. (2008). "The role of rearrangement at the second Ig heavy chain locus in maintaining B cell tolerance to DNA." J Immunol **180**(11): 7721–7.
- Loder, F., B. Mutschler, et al. (1999). "B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals." J Exp Med **190**(1): 75–89.
- Lu, R., K. L. Medina, et al. (2003). "IRF-4,8 orchestrate the pre-B-to-B transition in lymphocyte development." Genes Dev **17**(14): 1703–8.
- Luhder, F., P. Hoglund, et al. (1998). "Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes." J Exp Med **187**(3): 427–32.
- Lundholm, M., V. Motta, et al. (2006). "Defective induction of CTLA-4 in the NOD mouse is controlled by the NOD allele of Idd3/IL-2 and a novel locus (Ctex) telomeric on chromosome 1." Diabetes **55**(2): 538–44.

- Ma, S., A. Turetsky, et al. (2006). "IFN regulatory factor 4 and 8 promote Ig light chain kappa locus activation in pre-B cell development." J Immunol **177**(11): 7898–904.
- Mackay, F., S. A. Woodcock, et al. (1999). "Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations." J Exp Med **190**(11): 1697–710.
- Manning, B. D. and L. C. Cantley (2007). "AKT/PKB signaling: navigating downstream." Cell **129**(7): 1261–74.
- Marshall, A. J., H. E. Fleming, et al. (1998). "Modulation of the IL-7 dose-response threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression." J Immunol **161**(11): 6038–45.
- Martin, F. and J. F. Kearney (2000). "Positive selection from newly formed to marginal zone B cells depends on the rate of clonal production, CD19, and btk." Immunity **12**(1): 39–49.
- Martin, F., A. M. Oliver, et al. (2001). "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." Immunity **14**(5): 617–29.
- Mathieu, N., W. M. Hempel, et al. (2000). "Chromatin remodeling by the T cell receptor (TCR)-beta gene enhancer during early T cell development: Implications for the control of TCR-beta locus recombination." J Exp Med **192**(5): 625–36.
- Max, E. E., J. G. Seidman, et al. (1979). "Sequences of five potential recombination sites encoded close to an immunoglobulin kappa constant region gene." Proc Natl Acad Sci U S A **76**(7): 3450–4.
- McCormack, W. T., M. Liu, et al. (1993). "Excision products of TCR V alpha recombination contain in-frame rearrangements: evidence for continued V(D)J recombination in TCR+ thymocytes." Int Immunol **5**(7): 801–4.
- McMurry, M. T. and M. S. Krangel (2000). "A role for histone acetylation in the developmental regulation of VDJ recombination." Science **287**(5452): 495–8.
- Meixlsperger, S., F. Kohler, et al. (2007). "Conventional light chains inhibit the autonomous signaling capacity of the B cell receptor." Immunity **26**(3): 323–33.
- Merrell, K. T., R. J. Benschop, et al. (2006). "Identification of anergic B cells within a wild-type repertoire." Immunity **25**(6): 953–62.
- Meyer, K. B. and M. S. Neuberger (1989). "The immunoglobulin kappa locus contains a second, stronger B-cell-specific enhancer which is located downstream of the constant region." Embo J **8**(7): 1959–64.
- Miller, J. P., D. Izon, et al. (2002). "The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7." J Exp Med **196**(5): 705–11.

- Monroe, R. J., F. Chen, et al. (1999). "RAG2 is regulated differentially in B and T cells by elements 5' of the promoter." Proc Natl Acad Sci U S A **96**(22): 12713–8.
- Moore, M. W., J. Durdik, et al. (1985). "Deletions of kappa chain constant region genes in mouse lambda chain-producing B cells involve intrachromosomal DNA recombinations similar to V-J joining." Proc Natl Acad Sci U S A **82**(18): 6211–5.
- Mostoslavsky, R., N. Singh, et al. (1998). "Kappa chain monoallelic demethylation and the establishment of allelic exclusion." Genes Dev **12**(12): 1801–11.
- Muljo, S. A. and M. S. Schlissel (2003). "A small molecule Abl kinase inhibitor induces differentiation of Abelson virus-transformed pre-B cell lines." Nat Immunol **4**(1): 31–7.
- Muller, B. and M. Reth (1988). "Ordered activation of the Ig lambda locus in Abelson B cell lines." J Exp Med **168**(6): 2131–7.
- Mundt, C., S. Licence, et al. (2001). "Loss of precursor B cell expansion but not allelic exclusion in VpreB1/VpreB2 double-deficient mice." J Exp Med **193**(4): 435–45.
- Murakami, M., T. Tsubata, et al. (1992). "Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice." Nature **357**(6373): 77–80.
- Nakayama, M., N. Abiru, et al. (2005). "Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice." Nature **435**(7039): 220–3.
- Nemazee, D. A. and K. Burki (1989). "Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes." Nature **337**(6207): 562–6.
- Nishimoto, N., H. Kubagawa, et al. (1991). "Normal pre-B cells express a receptor complex of mu heavy chains and surrogate light-chain proteins." Proc Natl Acad Sci U S A **88**(14): 6284–8.
- Noorchashm, H., N. Noorchashm, et al. (1997). "B-cells are required for the initiation of insulinitis and sialitis in nonobese diabetic mice." Diabetes **46**(6): 941–6.
- Novobrantseva, T., S. Xu, et al. (2005). "Stochastic pairing of Ig heavy and light chains frequently generates B cell antigen receptors that are subject to editing in vivo." Int Immunol **17**(4): 343–50.
- Nutt, S. L., B. Heavey, et al. (1999). "Commitment to the B-lymphoid lineage depends on the transcription factor Pax5." Nature **401**(6753): 556–62.
- Ohnishi, K. and F. Melchers (2003). "The nonimmunoglobulin portion of lambda5 mediates cell-autonomous pre-B cell receptor signaling." Nat Immunol **4**(9): 849–56.
- Oliver, A. M., F. Martin, et al. (1999). "IgM<sup>high</sup>CD21<sup>high</sup> lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells." J Immunol **162**(12): 7198–207.

- Parker, M. J., S. Licence, et al. (2005). "The pre-B-cell receptor induces silencing of VpreB and lambda5 transcription." Embo J **24**(22): 3895–905.
- Pathak, S., S. Ma, et al. (2008). "A role for interferon regulatory factor 4 in receptor editing." Mol Cell Biol **28**(8): 2815–24.
- Pelanda, R., U. Braun, et al. (2002). "B cell progenitors are arrested in maturation but have intact VDJ recombination in the absence of Ig-alpha and Ig-beta." J Immunol **169**(2): 865–72.
- Pelanda, R., S. Schwes, et al. (1997). "Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification." Immunity **7**(6): 765–75.
- Persiani, D. M., J. Durdik, et al. (1987). "Active lambda and kappa antibody gene rearrangement in Abelson murine leukemia virus-transformed pre-B cell lines." J Exp Med **165**(6): 1655–74.
- Peschon, J. J., P. J. Morrissey, et al. (1994). "Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice." J Exp Med **180**(5): 1955–60.
- Picard, D. and W. Schaffner (1984). "A lymphocyte-specific enhancer in the mouse immunoglobulin kappa gene." Nature **307**(5946): 80–2.
- Pongubala, J. M., D. L. Northrup, et al. (2008). "Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5." Nat Immunol **9**(2): 203–15.
- Prak, E. L., M. Trounstein, et al. (1994). "Light chain editing in kappa-deficient animals: a potential mechanism of B cell tolerance." J Exp Med **180**(5): 1805–15.
- Prak, E. L. and M. Weigert (1995). "Light chain replacement: a new model for antibody gene rearrangement." J Exp Med **182**(2): 541–8.
- Prochazka, M., D. V. Serreze, et al. (1992). "NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice." Diabetes **41**(1): 98–106.
- Quinn, W. J., 3rd, N. Noorchashm, et al. (2006). "Cutting edge: impaired transitional B cell production and selection in the nonobese diabetic mouse." J Immunol **176**(12): 7159–64.
- Radic, M. Z., J. Erikson, et al. (1993). "B lymphocytes may escape tolerance by revising their antigen receptors." J Exp Med **177**(4): 1165–73.
- Ramsden, D. A. and G. E. Wu (1991). "Mouse kappa light-chain recombination signal sequences mediate recombination more frequently than do those of lambda light chain." Proc Natl Acad Sci U S A **88**(23): 10721–5.
- Reth, M., E. Petrac, et al. (1987). "Activation of V kappa gene rearrangement in pre-B cells follows the expression of membrane-bound immunoglobulin heavy chains." Embo J **6**(11): 3299–305.

- Retter, M. W. and D. Nemazee (1998). "Receptor editing occurs frequently during normal B cell development." J Exp Med **188**(7): 1231-8.
- Ridgway, W. M. (2006). "Dissecting genetic control of autoimmunity in NOD congenic mice." Immunol Res **36**(1-3): 189-95.
- Rieck, M., A. Arechiga, et al. (2007). "Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes." J Immunol **179**(7): 4704-10.
- Ritchie, K. A., R. L. Brinster, et al. (1984). "Allelic exclusion and control of endogenous immunoglobulin gene rearrangement in kappa transgenic mice." Nature **312**(5994): 517-20.
- Rolink, A. G., J. Andersson, et al. (1998). "Characterization of immature B cells by a novel monoclonal antibody, by turnover and by mitogen reactivity." Eur J Immunol **28**(11): 3738-48.
- Rolink, A. G., T. Winkler, et al. (2000). "Precursor B cell receptor-dependent B cell proliferation and differentiation does not require the bone marrow or fetal liver environment." J Exp Med **191**(1): 23-32.
- Roschenthaler, F., T. Kirschbaum, et al. (1999). "The 5' part of the mouse immunoglobulin kappa locus." Eur J Immunol **29**(7): 2065-71.
- Rowley, R. B., A. L. Burkhardt, et al. (1995). "Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Ig alpha/Ig beta immunoreceptor tyrosine activation motif binding and autophosphorylation." J Biol Chem **270**(19): 11590-4.
- Rumfelt, L. L., Y. Zhou, et al. (2006). "Lineage specification and plasticity in CD19- early B cell precursors." J Exp Med **203**(3): 675-87.
- Santulli-Marotto, S., M. W. Retter, et al. (1998). "Autoreactive B cell regulation: peripheral induction of developmental arrest by lupus-associated autoantigens." Immunity **8**(2): 209-19.
- Schlissel, M. S. (2004). "Regulation of activation and recombination of the murine Igkappa locus." Immunol Rev **200**: 215-23.
- Schlissel, M. S. and D. Baltimore (1989). "Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription." Cell **58**(5): 1001-7.
- Schram, B. R., L. E. Tze, et al. (2008). "B cell receptor basal signaling regulates antigen-induced Ig light chain rearrangements." J Immunol **180**(7): 4728-41.
- Sekiguchi, D. R., L. Yunk, et al. (2006). "Development and selection of edited B cells in B6.56R mice." J Immunol **176**(11): 6879-87.
- Serreze, D. V., H. D. Chapman, et al. (1996). "B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new "speed congenic" stock of NOD.Ig mu null mice." J Exp Med **184**(5): 2049-53.

- Shlomchik, M. J., M. P. Madaio, et al. (1994). "The role of B cells in lpr/lpr-induced autoimmunity." J Exp Med **180**(4): 1295–306.
- Sikes, M. L., A. Meade, et al. (2002). "Regulation of V(D)J recombination: a dominant role for promoter positioning in gene segment accessibility." Proc Natl Acad Sci U S A **99**(19): 12309–14.
- Silveira, P. A., H. D. Chapman, et al. (2006). "Genes within the Idd5 and Idd9/11 diabetes susceptibility loci affect the pathogenic activity of B cells in nonobese diabetic mice." J Immunol **177**(10): 7033–41.
- Silveira, P. A., J. Dombrowsky, et al. (2004). "B cell selection defects underlie the development of diabetogenic APCs in nonobese diabetic mice." J Immunol **172**(8): 5086–94.
- Siminovitch, K. A., A. Bakhshi, et al. (1985). "A uniform deleting element mediates the loss of kappa genes in human B cells." Nature **316**(6025): 260–2.
- Siminovitch, K. A., M. W. Moore, et al. (1987). "The human kappa deleting element and the mouse recombining segment share DNA sequence homology." Nucleic Acids Res **15**(6): 2699–705.
- Skok, J. A., K. E. Brown, et al. (2001). "Nonequivalent nuclear location of immunoglobulin alleles in B lymphocytes." Nat Immunol **2**(9): 848–54.
- Stanhope-Baker, P., K. M. Hudson, et al. (1996). "Cell type-specific chromatin structure determines the targeting of V(D)J recombinase activity in vitro." Cell **85**(6): 887–97.
- Su, I. H., A. Basavaraj, et al. (2003). "Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement." Nat Immunol **4**(2): 124–31.
- Taccioli, G. E., T. M. Gottlieb, et al. (1994). "Ku80: product of the XRCC5 gene and its role in DNA repair and V(D)J recombination." Science **265**(5177): 1442–5.
- Taccioli, G. E., G. Rathbun, et al. (1993). "Impairment of V(D)J recombination in double-strand break repair mutants." Science **260**(5105): 207–10.
- Takaishi, H., H. Konishi, et al. (1999). "Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B." Proc Natl Acad Sci U S A **96**(21): 11836–41.
- Takeda, S., Y. R. Zou, et al. (1993). "Deletion of the immunoglobulin kappa chain intron enhancer abolishes kappa chain gene rearrangement in cis but not lambda chain gene rearrangement in trans." Embo J **12**(6): 2329–36.
- Tamura, T., H. Yanai, et al. (2008). "The IRF family transcription factors in immunity and oncogenesis." Annu Rev Immunol **26**: 535–84.
- Tan, E. M. (1982). "Autoantibodies to nuclear antigens (ANA): their immunobiology and medicine." Adv Immunol **33**: 167–240.



- Tan, E. M., A. S. Cohen, et al. (1982). "The 1982 revised criteria for the classification of systemic lupus erythematosus." Arthritis Rheum **25**(11): 1271-7.
- Thiebe, R., K. F. Schable, et al. (1999). "The variable genes and gene families of the mouse immunoglobulin kappa locus." Eur J Immunol **29**(7): 2072-81.
- Thien, M., T. G. Phan, et al. (2004). "Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches." Immunity **20**(6): 785-98.
- Thompson, E. C., B. S. Cobb, et al. (2007). "Ikaros DNA-binding proteins as integral components of B cell developmental-stage-specific regulatory circuits." Immunity **26**(3): 335-44.
- Tiegs, S. L., D. M. Russell, et al. (1993). "Receptor editing in self-reactive bone marrow B cells." J Exp Med **177**(4): 1009-20.
- Tokoyoda, K., T. Egawa, et al. (2004). "Cellular niches controlling B lymphocyte behavior within bone marrow during development." Immunity **20**(6): 707-18.
- Tsuiji, M., S. Yurasov, et al. (2006). "A checkpoint for autoreactivity in human IgM+ memory B cell development." J Exp Med **203**(2): 393-400.
- Turner, M., P. J. Mee, et al. (1995). "Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk." Nature **378**(6554): 298-302.
- Tze, L. E., B. R. Schram, et al. (2005). "Basal immunoglobulin signaling actively maintains developmental stage in immature B cells." PLoS Biol **3**(3): e82.
- van der Burg, M., B. H. Barendregt, et al. (2002). "Immunoglobulin light chain gene rearrangements display hierarchy in absence of selection for functionality in precursor-B-ALL." Leukemia **16**(8): 1448-53.
- Vang, T., M. Congia, et al. (2005). "Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant." Nat Genet **37**(12): 1317-9.
- Vela, J. L., D. Ait-Azzouzene, et al. (2008). "Rearrangement of mouse immunoglobulin kappa deleting element recombining sequence promotes immune tolerance and lambda B cell production." Immunity **28**(2): 161-70.
- Wang, D., J. Feng, et al. (2000). "Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors." Immunity **13**(1): 25-35.
- Wardemann, H., S. Yurasov, et al. (2003). "Predominant autoantibody production by early human B cell precursors." Science **301**(5638): 1374-7.
- Watanabe-Fukunaga, R., C. I. Brannan, et al. (1992). "Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis." Nature **356**(6367): 314-7.
- Wei, X. C., H. Kishi, et al. (2002). "Characterization of chromatin structure and enhancer elements for murine recombination activating gene-2." J Immunol **169**(2): 873-81.

- Whitehurst, C. E., M. S. Schlissel, et al. (2000). "Deletion of germline promoter PD beta 1 from the TCR beta locus causes hypermethylation that impairs D beta 1 recombination by multiple mechanisms." Immunity **13**(5): 703–14.
- Wicker, L. S., G. Chamberlain, et al. (2004). "Fine mapping, gene content, comparative sequencing, and expression analyses support Ctla4 and Nramp1 as candidates for Idd5.1 and Idd5.2 in the nonobese diabetic mouse." J Immunol **173**(1): 164–73.
- Witsch, E. J., H. Cao, et al. (2006). "Light chain editing generates polyreactive antibodies in chronic graft-versus-host reaction." J Exp Med **203**(7): 1761–72.
- Xu, C. R. and A. J. Feeney (2009). "The epigenetic profile of Ig genes is dynamically regulated during B cell differentiation and is modulated by pre-B cell receptor signaling." J Immunol **182**(3): 1362–9.
- Xu, Y., L. Davidson, et al. (1996). "Deletion of the Ig kappa light chain intronic enhancer/matrix attachment region impairs but does not abolish V kappa J kappa rearrangement." Immunity **4**(4): 377–85.
- Yamanouchi, J., D. Rainbow, et al. (2007). "Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity." Nat Genet **39**(3): 329–37.
- Yancopoulos, G. D. and F. W. Alt (1985). "Developmentally controlled and tissue-specific expression of unrearranged VH gene segments." Cell **40**(2): 271–81.
- Yancopoulos, G. D., B. A. Malynn, et al. (1988). "Developmentally regulated and strain-specific expression of murine VH gene families." J Exp Med **168**(1): 417–35.
- Yu, W., Z. Misulovin, et al. (1999). "Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5' of RAG2." Science **285**(5430): 1080–4.
- Yunk, L., W. Meng, et al. (2009). "Antibodies in a heavy chain knock-in mouse exhibit characteristics of early heavy chain rearrangement." J Immunol **183**(1): 452–61.
- Yurasov, S., H. Wardemann, et al. (2005). "Defective B cell tolerance checkpoints in systemic lupus erythematosus." J Exp Med **201**(5): 703–11.
- Zarrin, A. A., I. Fong, et al. (1997). "Cloning and characterization of the human recombination activating gene 1 (RAG1) and RAG2 promoter regions." J Immunol **159**(9): 4382–94.
- Zhang, J., V. Roschke, et al. (2001). "Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus." J Immunol **166**(1): 6–10.
- Ziegler, R., C. A. Alper, et al. (1991). "Specific association of HLA-DR4 with increased prevalence and level of insulin autoantibodies in first-degree relatives of patients with type I diabetes." Diabetes **40**(6): 709–14.
- Zou, Y. R., S. Takeda, et al. (1993). "Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa." Embo J **12**(3): 811–20.